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Studies in Vernalisation

XII. The Reversibility by High Temperature of the Vernalised Condition in Petkus Winter Rye

BY

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With one Figure in the Text

ABSTRACT

The reversibility of vernalisation in Petkus winter rye has been studied in detail, by using alternating periods, during germination, of low and high temperature of varying durations. The degree of devernalisation by exposure to subsequent high temperature varies with the initial duration of vernalisation and becomes progressively less as vernalisation proceeds. A quantitative method of studying these effects is described, and a revised schema of the dynamic aspects of vernalisation put forward.

I. INTRODUCTION

EVIDENCE has been presented in a former paper of this series (Studies, XI) indicating that even with a constant duration of vernalisation the effect on flowering behaviour varies considerably from year to year. These seasonal factors must exert their influence after the vernalisation treatment is over. Exposure to low temperature after planting out will by a 'natural vernalisation' intensify the effect of previous vernalisation (so long as this is not complete), but other factors may partially annul the effects and delay flowering. The question as to the stability of the vernalised condition of the grain thus arises. The theory of phasic development of Lysenko (1932) assumed that the thermophase is non-reversible; indeed, according to this theory the 'thermostage' must be traversed before further development and entry into the 'photophase' can take place. Experiments varying the duration of vernalisation have proved beyond doubt that the thermostage need not be completed in order to ensure later flowering, and the time to flower is determined by the degree of vernalisation attained.

There is considerable evidence that the vernalisation effect can be reversed: thus Chroboczek (1934), Owen, Carsner, and Stout (1940), and others have found with beet (*Beta vulgaris*) and Lang and Melchers (1947) with *Hyoscyamus*

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that a period of high temperature following upon the vernalisation treatment annuls the accelerating effect in flowering induced by low temperature. Again, in cereals Efeikin (1941) and Tetjurev (1941) have demonstrated with winter wheat a complete devernalisation of vernalised grain by exposure to 35° C. for 3 days. In 1938 (Studies, III) Gregory and Purvis showed with Petkus winter rye that with a fixed duration of low temperature of 1° C. for 6 weeks the effect on flowering could be varied by interspersing periods at 20° C. during the exposure to low temperature. Thus, whereas an unbroken period of 6 weeks at 1° C. induced flowering, with alternate days at 1° C. and 20° C. (total duration of low temperature 6 weeks) the grain remained unvernalised. When the proportions of days at 20° C. and 1° C. were changed to 1 in 3, 1 in 4, 1 in 7, a gradation in time to flowering was obtained. On the other hand, Sen (1946) found with mustard (*Sinapis alba*) that vernalisation was quite unaffected by subsequent exposure to high temperature.

A further experiment using Petkus winter rye (Gregory and Purvis, 1945) showed that high-temperature treatment following vernalisation certainly partially annulled the effect, but complete reversal as recorded by Efeikin and Tetjurev did not occur. The suggestion was, however, put forward (Gregory and Purvis, 1948) that the reversal by high temperature varies with the degree of vernalisation and the vernalised condition becomes more and more stable the longer the low temperature is maintained. The apparent discrepancies in the findings of different workers might thus be resolved.

Two separate problems must be distinguished, namely: (1) the effect of high temperatures during the course of vernalisation, i.e. alternating periods of high and low during vernalisation, and (2) the effect of high temperature after the grain has been exposed to an unbroken period of low temperature.

In the experiments described in this paper both of these problems are dealt with.

II. THE DEVERNALISING EFFECT OF SHORT PERIODS AT 20° C. INTERPOLATED IN THE VERNALISATION PERIOD

(*a*) *Effect of alternating periods at low and high temperatures before sowing*

In the experiment described above in which imbibed rye grain was exposed alternately to high and low temperatures, anaerobic conditions were imposed during the warm period in order to check excessive growth after germination. Since this technique has been criticized and the validity of the results questioned (Bassarskaya and Grossman, 1941) the experiment was repeated in 1946 when the grain was freely exposed to air and growth was checked by maintaining a low moisture level. The correct moisture level (50 per cent. of the absolute dry weight) was maintained without difficulty during the periods at 20° C. by spraying the carefully sterilized grain with boiled and cooled tap-water to a predetermined weight. The whole operation was carried out in 3-in. glass dishes with lids, as described on p. 8.

All the series received a total exposure of 42 days at low temperature

(1·8° C.). Twenty-four hour periods at 20° C. were interpolated (*a*) every alternate day, (*b*) every third day, (*c*) every fourth day, and (*d*) every seventh day. A control series was kept for 42 consecutive days at 1·8° C. and a second control series was sterilized and kept for 4 days at 20° C. and 50 per cent. moisture without vernalisation treatment. These treatments were timed to allow simultaneous sowing on May 7, 1946. The seed was sown thinly in 1-in. drills, 6 ft. long and 11 in. apart, and thinned to 2-in. spacing on June 7.

The results are entered as 'scores' in Table I—the higher the 'score' the more rapid was progress towards flowering.

TABLE I

Effect of Interpolated 24-hour Periods at 20° C. on the Flowering of Winter Rye vernalised 6 Weeks. Sown in the field May 7, 1946

Ratio $\frac{\text{Time at 1·8° C.}}{\text{Time at 20° C.}}$	0/0	1/1	2/1	3/1	6/1	6/0
Number of replicates	(33)	(33)	(41)	(40)	(49)	(61)
'Score' after 133 days	23±0·8	41±6·2	64±6·2	85±5·5	100±3·7	120±0·6
Differences . .		18±6·3	23±8·8	21±8·3	15±6·5	20±3·4

The mean 'scores' show that the degree of vernalisation attained in 6 weeks at low temperature is progressively reduced by the increasing number of interpolated periods at 20° C. Equal exposures to high and low temperature do here result in a small degree of vernalisation. Otherwise the results obtained in 1937, using the anaerobic check on growth, are confirmed.

(*b*) *Effect of varying the duration of the alternate periods of high and low temperature during vernalisation*

The possibility was envisaged that the sensitivity to high temperature might vary with the stage of vernalisation. This possibility was tested in the following simple way. The relative durations of the periods of low and high temperature were maintained constant at a ratio of 2:1, but the absolute lengths were changed. Appropriate controls were used. The experiment was carried out with Petkus winter rye in 1948. Grain was sterilized and prepared for vernalisation, and the following treatments given:

1. Continuous vernalisation for 6 weeks at 1·8° C. followed by 3 weeks at 20° C., during which germination continued to be checked by controlling the moisture content.
2. Two periods of 3 weeks at 1·8° C. each followed by 10½ days at 20° C.
3. Three periods of 2 weeks at 1·8° C. each followed by 1 week at 20° C.
4. Seven periods of 6 days at 1·8° C. each followed by 3 days at 20° C.
5. Twenty-one 2-day periods at 1·8° C. each followed by 24 hours at 20° C.
6. Control series vernalised 6 weeks without any high-temperature period.
7. Control series unvernalised.

The results set out in Table II show that the more numerous the cycles the less the degree of vernalisation attained.

TABLE II

Flowering Behaviour of Winter Rye vernalised for a Total Period of 6 Weeks, with Interpolated Periods at 20° C. totalling 3 Weeks, and varied in Duration from 1 Day to 3 Weeks. Sown in the Field and in Pots, May 5, 1948

Length of cold periods in days . . .	42	42	21	14	6	2	0
Length of warm periods in days . . .	0	21	10½	7	3	1	0
No. of cycles . .	— (control)	1	2	3	7	21	— (control)
'Score', field culture .	88±3·5	100±2·1	78±4·4	42±4·8	34±3·9	30±3·7	26±0·7
No. of replicates . .	(51)	(69)	(51)	(27)	(29)	(36)	(59)
'Score', pot culture .	107±2·0	111±0·8	99±6·0	67±11·6	46±6·4	35±7·4	25±1·5
No. of replicates . .	(12)	(14)	(14)	(12)	(11)	(12)	(11)

In the series with unbroken 6 weeks of vernalisation followed by 3 weeks at 20° C. the 'score' was actually higher than in the control also vernalised for 6 weeks but without high temperature following after. This result was probably due to the fact that some progress in growth was made during the 21 days at 20° C. despite the low moisture content of the grain.

The experiment demonstrates three important facts: first, that temperature as low as 20° C. will reverse the low-temperature effect; second, that repeated short exposures to high temperature are more effective than fewer exposures of long duration, the total period at low temperature remaining constant. The third fact disclosed by the experiment is that after 6 weeks of vernalisation, a temperature of 20° C. fails to reverse the process. When the subsequent high temperature is raised to 35° C., reversal after 6 weeks' vernalisation is found, as the following experiments show.

III. THE EFFECT OF EXPOSURE TO HIGH TEMPERATURE AFTER AN UNINTERRUPTED PERIOD OF VERNALISATION

In 1944 a comprehensive experiment with Petkus rye was carried out to determine how far the results of Efeikin (1939) and Tetjurev (1940) with winter wheat applied to rye.

Method

Both spring and winter Petkus rye were used, the latter vernalised for 6 weeks at 1° C. and both subjected to a range of high temperatures for periods varying from 2 hours to 5 days.

During vernalisation Efeikin (1941) and Tetjurev (1941) restricted the water supplied to the grain (Russian technique), whereas in previous work by the authors the water-supply to the grain was not restricted, as the seeds were kept in well-drained wet sand. To cover all contingencies both methods were used in the experiment of 1944 to be described. Some of the results have already appeared in 'Nature' (Gregory and Purvis, 1945); all the data are now given.

The method of unrestricted water-supply has certain advantages: thus, first, it obviates the need for repeated weighing during vernalisation to replace the water lost by evaporation (in the Russian technique accurate control of water content is essential, as below a critical water content vernalisation fails). Second, at the end of the vernalisation period, by immersing the seed boxes with the sand several times in water at 40° C. the temperature could be raised rapidly to the required high temperature and the beginning of the heat treatment thus be accurately timed, a matter of importance in short treatments. The temperature aimed at was 35° C., and after the sand had reached this temperature (10 to 15 minutes) the boxes were placed in a thermostat at 35° C. Owing, however, to cooling by evaporation the temperature of the sand varied from 26 to 30° C. The times of heat treatment in this series were 2, 4, 8 hours, and 1, 2, 3 days respectively. The results for spring and winter rye are given in Table III.

TABLE III

Effect of High Temperature on Spring Rye and on Winter Rye vernalised for 6 Weeks. Vernalised on Sand. Sown in Field, May 8–12, 1944. Number of Replicates in brackets

Duration of exposure to heat (26–30° C.).	Unvernalised spring rye. 'Scores'	Winter rye vernalised 6 weeks. 'Scores'
0	123±0·5 (54)	114±2·7 (38)
2 hours	118±0·8 (41)	110±0·8 (79)
4 hours	117±1·0 (49)	111±2·9 (32)
8 hours	119±0·9 (26)	110±1·7 (34)
1 day	117±0·9 (47)	101±2·1 (69)
2 days	121±0·8 (54)	97±2·7 (86)
3 days	121±0·8 (21)	94±3·8 (49)
	Unvernalised winter rye	22±1·6 (27)

In the other series the 'Russian technique' with restricted water was used. After determining the absolute dry weight of the grain, 5-g. lots of the air-dry grain were placed in shallow glass dishes (3-in. diam.) of known weight and provided with lids. Fifty cubic centimetres of calcium hypochlorite solution equivalent to 1 per cent. chlorine (Wilson, 1920) were added to each dish, and after stirring to remove air bubbles the dishes were left at 20° C. for 4 hours and twice washed in sterilized distilled water, and allowed to soak for a further 2 hours. Surplus water was removed and the moist grain was replaced in the dish. The water content of the grain was adjusted accurately to the required 50 per cent. by spraying with sterile water from an atomizer. After 24 hours at 20° C. the dishes were transferred to a low-temperature room (1° C.) for 6 weeks, and twice weekly were sprayed to maintain the water content of the grain at between 50 and 55 per cent. of the dry weight. At the end of vernalisation for 6 weeks the dishes were placed in a thermostat at 35° C. for periods of 8, 16 hours, and 1–5 days. Daily the water content was adjusted by weighing. The results are shown in Table IV.

TABLE IV

Effect of Exposure to 35° C. on Spring Rye and on Winter Rye vernalised for 6 Weeks. Restricted Moisture Technique. Sown in Field, May 8–12, 1944. Number of Replicates in brackets

Duration of heat treatment.	Unvernalised spring rye. 'Scores'	Winter rye vernalised 6 weeks. 'Scores'
0	123±0·1 (52)	100±3·0 (47)
8 hours	120±0·8 (48)	95±4·6 (44)
16 ,,	121±0·9 (53)	90±4·9 (35)
1 day	120±0·7 (62)	75±6·5 (35)
2 days	121±0·7 (47)	80±5·9 (39)
3 ,,	124±0·4 (55)	72±9·5 (16)
4 ,,	123±0·5 (49)	72±6·4 (33)
5 ,,	120±0·6 (42)	63±6·6 (35)
	Unvernalised winter rye	22±0·6 (40)

Results

The data in Tables III and IV show the results of heat treatment on winter rye vernalised for 6 weeks and on unvernalised spring rye. The grain was planted out in the field on May 8–12, 1944. The results are again entered as 'scores'.

The flowering of spring rye is little affected by the heat treatment: such variations in 'score' as are present are unrelated to the period of heat treatment. The data with the 'Russian technique' (Table IV) show a lower 'score' for the vernalised control set, and also a more complete devernalisation by heat than the data in Table III with unrestricted water-supply to the grain. With vernalised winter rye a marked and progressive devernalisation appears as the heat treatment is prolonged.

Analysis of variance shows that there is no evidence that the relation between devernalisation and duration of heat treatment is other than linear. The effect is highly significant. Devernalisation is far from complete, as the differences from the 'score' of the unvernalised control show.

The lower 'scores' in the vernalised winter rye after heat treatment are not due to any injurious effect of the high temperature, as is indicated by the behaviour of the spring rye, for in this series even 5 days of heat treatment has scarcely retarded flowering. After this drastic treatment a high proportion of the grains succumbed, but the survivors grew quite normally. It will be shown later that the 'devernalised' winter rye grain can be revernalised and shows no deleterious after-effect of the heat treatment. Among the sets of heat-treated grains some individual plants showed no devernalisation, and in consequence the variability of the 'scores' increases as the heat treatment is increased, as appears from the increasing standard errors of the mean 'scores' of the winter rye, whereas the spring rye shows no such consistent variability.

IV. DEVERNALISATION BY HEAT AFTER VARYING DURATION OF PREVIOUS VERNALISATION TREATMENT

(*a*) *Experiments of 1945 and 1946*

Experiments to determine the relation between duration of vernalisation and the effect of subsequent heat treatment were carried out in 1945 and 1946. Effort was concentrated on ensuring a more precise control of temperature during the heat treatment while using an unrestricted water-supply. The end in view was to obtain data sufficiently precise to enable estimates to be made of the dynamics of the devernalisation process. Accordingly, in 1945 the sterilized grain was sown separately in test tubes, $5 \times \frac{5}{8}$ in., containing 10 g. of washed sand and 2 c.c. of water. The tubes were previously plugged with cotton-wool and autoclaved. Twenty-four hours after sowing the tubes were kept for the specified time at 1° C. and then immersed above the sand level in water in a thermostat kept at 35° C. Less than 1 minute sufficed to bring the temperature of the sand to the same level. The water in the thermostat was kept circulating round the tubes throughout the time of immersion.

The method used unfortunately proved unsuitable as vernalisation was poor and variable and so the effect of heat treatment could not be assessed. Considerable further work was carried out to investigate the cause of the partial failure of vernalisation, but the matter has not been entirely cleared up.

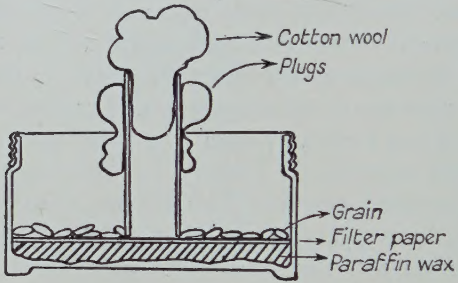
In the experiment of 1946 covered glass dishes (13 cm. diameter) were used, each containing 120 sterilized seeds sown under about half an inch of sand. As the seedlings grew, the covers were raised on sterile collars. In the saturated atmosphere in the dishes the seedlings grew during vernalisation to a much larger size than in the seed boxes previously used. There is reason to believe that the greater growth made had an important and unsuspected effect on subsequent devernalisation by high temperature, for in this experiment very little devernalisation occurred. It appears therefore that the devernalisation depends upon the stage of growth reached by the plants. The matter is discussed later.

The results of the experiment of 1945 are entered in Table V, in which the retardation after varying periods of vernalisation and heat treatment in conventional 'score' units is shown.

Owing to the variability in vernalisation in this experiment, as already mentioned, the standard errors are very high and the results erratic. It will, however, be noted that the degree of devernalisation decreases with the duration of the previous vernalisation treatment, and that the longer the vernalisation the longer the period of heat treatment required to devernalise the plants. With 12 weeks' vernalisation no reversal occurred. These results alone would scarcely suffice to establish the point as only one of the figures entered is statistically significant: fortunately a later experiment completely established the relationship.

(*b*) *Experiment of 1947*

In view of the difficulties encountered with the method of unrestricted water-supply, resort was again made to the 'Russian technique' in a further experiment. Glass jars, 7 cm. in diameter and 3 cm. high, provided with screw lids of metal, were used. The uneven floors of the jars were levelled by pouring in a layer of paraffin wax. The lids were perforated to carry a glass tube 1 cm. diam., which was supported by a wad of cotton-wool, and the upper end of the tube plugged. The arrangement is shown in the figure. The whole was sterilized in an oven and before use two layers of sterile filter-paper were inserted and moistened with 0·5 c.c. of sterilized water. A known weight of seed was sterilized, imbibed as described on p. 5 to 50 per cent. of the dry weight, and placed in each jar; care was taken that every seed was in contact with the moist paper. At intervals during the treatments sterile water was added through the central tube to maintain the moisture at the level stated. This arrangement has proved very successful.



TEXT-FIG.

After 24 hours at 20° C. the dishes were kept at 1·8° C. for the vernalisation period. During the heat treatment the dishes were kept in a saturated atmosphere which obviated further watering.

After treatment the seedlings were planted in soil on May 6 in triplicate 6-foot rows in a garden bed, and also in 11-in. earthenware pots in soil at the rate of 12 plants per pot. The results as 'scores' are entered in Table VI.

The 'scores' for the sets vernalised for the periods as stated in column 1 are entered in columns 2 and 3; the unheated controls are followed by the corresponding set heated for 3 days at 35° C. The acceleration in flowering due to vernalisation is shown in column 4, which constitutes a 'duration series', derived from the data in column 2. In column 5 are the estimates of devernalisation in 'score units' obtained by differences of corresponding 'scores' in columns 2 and 3. Finally, in column 6 are values of percentage

TABLE V

Retarding Effect of Heating to 35° C. in Relation to Duration of Previous Vernalisation Treatment. Sown in Field, April 30–May 4, 1945

Duration of vernalisation.	Retardation in conventional scoring units due to heating for 1 day.	2 days.	3 days.
20 days	5·8±5·9	6·8±6·7	9·3±6·3
25 ,,	0	11·3±8·7	18·9±7·3
30 ,,	4·1±10·7	9·8±9·7	−7·1±10·5
35 ,,	−8·2±10·4	11·2±9·5	21·0±12·1
42 ,,	−8·3±10·4	−15·0±10·5	6·8±11·7
84 ,,	1·1±1·2	2·3±1·4	1·1±1·4

TABLE VI

Effect of Exposure to 35° C. on Winter Rye vernalised with Restricted Moisture for Different Periods. 'Scores' in Conventional Units. Sown April 29–May 2, 1947. Number of Replicates in brackets

Duration of vernalisation.	Unheated.	Heated 3 days at 35° C.	Acceleration due to vernalisation.	Devernalisation.	% Devernalisation.
I. Field culture.					
0	24±1·4 (42)	24±0·9 (43)	0	—	—
2 weeks	28±2·6 (40)	24±1·3 (34)	3	3·2±3·1	100
3 ,,	62±4·4 (59)	41±3·9 (45)	38	22·2±6·1	58
4 ,,	79±4·6 (56)	48±5·3 (44)	55	30·5±7·0	56
5 ,,	108±1·1 (42)	87±5·5 (38)	84	21·2±5·4	25
6 ,,	112±0·8 (48)	98±3·0 (49)	88	13·8±3·1	16
8 (heated 5 days)	121±0·5 (53)	118±1·0 (45)	97	3·0±1·1	3 (Sown May 6)
II. Pot culture.					
0	26±3·0 (5)	34±4·0 (6)	0	−8·2±5·2	—
2 weeks	58±10·9 (7)	31±4·1 (10)	32	27·0±10·3	84
3 ,,	89±6·2 (8)	69±9·5 (9)	63	20·4±11·7	32
4 ,,	113±2·5 (11)	96±5·0 (7)	87	17·1±4·4	20
6 ,,	118±1·5 (8)	114±4·7 (10)	92	4·3±5·4	5

devernalisation (values in column 5 as percentage of values in column 4). It is evident that the longer the duration of vernalisation the less the percentage devernalisation. It also appears that in the pot cultures the degree of vernalisation is greater than for plants grown in soil similarly treated.

The analysis of the heat effect can be carried a little farther. In Table VII are entered the 'scores' for the earliest and latest plant in the replicates for any one vernalisation treatment, and also the percentage of plants in the sample which failed to reach the flowering stage (i.e. extrusion of anthers). Values for unheated and heat-treated sets are entered. Even among the unheated sets, unvernalised or given less than 4 weeks' vernalisation, some

TABLE VII

Highest and Lowest Individual 'Scores' of Sets vernalised for Varying Periods (whose Mean 'Scores' are entered in Table VI) unheated or subsequently heated for 3 Days at 35° C. The Percentages of Plants failing to flower in 133 Days are also entered

	Unheated.			Heated.		
Duration of vernalisation.	Earliest.	Latest.	% plants not flowering in 133 days.	Earliest.	Latest.	% plants not flowering in 133 days.
0	70	13	98	39	13	100
2 weeks	80	11	90	56	11	97
3 ,,	114	11	42	96	15	71
4 ,,	118	16	25	109	14	68
5 ,,	120	83	0	120	15	18
6 ,,	121	97	0	120	23	6
8 ,,	127	107	0	127	98	0
Spring rye	131	121	0	—	—	—

individual plants failed to reach the flowering condition, and among the heat-treated even after 6 weeks of vernalisation some such plants occur. With vernalisation of 2–4 weeks the main effect of the heat treatment is on the early plants with maximal 'score', since those with minimal 'scores' failed to be vernalised and therefore heat can have had no reversing effect. With vernalisation greater than 4 weeks the earliest plants with maximal 'scores' are not affected by heat treatment, for in these vernalisation has become fixed; the less effectively vernalised individuals now show the retardation by heat.

TABLE VIII

The Effect of Exposure to 35° C. on Leaf Production on Main Axis of Plants vernalised for Different Periods. Sown in Pots, April 29–May 2, 1947. Number of Replicates in brackets

Duration of vernalisation.	Unheated.	Heated.	Difference.	
0	21·8±0·6 (5)	21·2±0·8 (6)	0·6±1·0	ns
2 weeks	19·2±1·3 (7)	22·3±0·7 (10)	3·1±1·3	s
3 ,,	16·2±0·6 (8)	19·8±0·9 (9)	3·6±1·1	ss
4 ,,	12·7±0·5 (11)	18·0±1·6 (7)	5·3±1·4	ss
6 ,,	10·8±0·5 (8)	11·8±0·7 (10)	1·0±0·9	ns

Significance: ns, $P > 0{\cdot}05$; s, $P < 0{\cdot}05$; ss, $P < 0{\cdot}01$

The number of leaves on the main axis of the pot plants are given in Table VIII. The delay in flowering occasioned by heat treatment is thus accompanied by an increase in leaf number, indicating beyond doubt that a real reversal of vernalisation is concerned and no mere injurious effect.

As seen from the data in Table IX, heat treatment after vernalisation also affects the production of tillers. The values entered are the means of the

TABLE IX

Effect of Exposure to 35° C. on Tiller Production in vernalised Winter Rye. Means of 'Peak' Tiller Numbers. Sown in Pots, April 29–May 2, 1947

Duration of vernalisation.	Peak tiller numbers. Unheated.	Heated.	Increase due to heat.	
0	40·7 ± 3·6	28·8 ± 2·9	−11·9 ± 4·9	s
2 weeks	39·6 ± 3·5	34·0 ± 2·7	−5·6 ± 4·5	ns
3 ,,	23·2 ± 2·5	29·7 ± 2·9	+6·5 ± 3·8	ns
4 ,,	20·5 ± 2·5	28·8 ± 1·6	+8·3 ± 3·0	ss
6 ,,	12·1 ± 1·6	12·6 ± 1·9	+0·5 ± 2·4	ns

Significance: ns, $P > 0·05$; s, $P < 0·05$; ss, $P < 0·01$
Each value is mean of 12 plants.

maximum tiller number of each plant at the peak of its vegetative development. Heat treatment has significantly reduced the tiller number in unvernalised grain, but with increasing periods of vernalisation the decrease is diminished and gives place to an increase in vegetative development. This effect may be due merely to delay in flowering, but is not incompatible with a transformation of a flower-forming substance into a vegetative hormone such as has been previously postulated (Studies, II).

All the data so far presented show that with increasing duration of vernalisation the reversal by heat becomes progressively less. The stability of the vernalised condition appears to vary in different individuals, as the data in Table VII show. Thus the 'earliest' plants after 5 weeks of vernalisation show no reversal by subsequent heat. The values for the mean plant entered in Table VI show that the average plant vernalised for 8 weeks has almost reached the irreversible condition. Below, in Table X, are given 'scores' for plants vernalised for 12 weeks and then exposed for varying times to a temperature of 35° C.

TABLE X

Effect of Exposure to 35° C. on Winter Rye vernalised for 12 Weeks. Sown in Field, May 1–5, 1944. 'Scores' in Conventional Units

Duration at 35° C.	Control	16 hours	1 day	2 days	3 days	4 days	5 days
'Russian technique'	112 ± 1·2	114 ± 1·0	116 ± 1·2	115 ± 0·9	108 ± 3·3	116 ± 0·7	115 ± 0·7
Unrestricted water	122 ± 0·6	119 ± 0·6	120 ± 0·5	120 ± 0·6	118 ± 0·6	—	—

The vernalised condition is quite irreversible even after 5 days of high temperature.

V. THE EFFECT OF STAGE OF SEEDLING DEVELOPMENT ON DEVERNALISATION BY HEAT

The failure of devernalisation in the experiment of 1946 has been mentioned. The seedlings in this experiment were more than 3 in. long with the first leaf emerged. It is suggested that the size of the seedlings at the beginning of the heat treatment is of greater importance than during vernalisation. If this is so it should be possible to fix the vernalised condition by allowing growth to proceed for a short time at a neutral temperature before applying the heat treatment. Efeikin (1941) found in wheat that the vernalised condition was fixed after a certain stage in growth and heat devernalisation then failed. Lang and Melchers (1947) found with *Hyoscyamus niger* that such treatment prevents devernalisation by heat. A similar experiment was carried out with Petkus winter rye in 1947. After vernalisation for 4 and 6 weeks by the 'Russian technique', the seedlings were either treated at 35° C. immediately or after 2, 3, and 4 days at 17° C. Control series were treated in the same way without heat treatment. The results are entered in Table XI.

TABLE XI

Effect of Delaying Heat Treatment of Winter Rye Sown in Field, April 25–May 2, 1947. Number of Replicates in brackets

Days' growth at 17° C. in sand after vernalisation.	0	2	3	4	Diff. 0–4, i.e. devernalisation at 17° C
Vernalised 6 weeks					
Unheated . .	111±0·9 (43)	109±1·4 (30)	105±4·0 (25)	101±4·2 (23)	10±3·3
Heated 3 days . .	100±4·1 (22)	97±4·1 (31)	99±4·1 (32)	97±5·1 (19)	
Difference . .	10·9±3·2	11·9±4·9	6·4±5·8	4·1±6·5	
Significance . .	ss	s	ns	ns	ss
Vernalised 4 weeks					
Unheated . .	84±4·8 (35)	53±6·9 (26)	68±7·1 (29)	52±7·7 (17)	32±8·7
Heated 3 days . .	43±7·2 (20)	38±5·3 (25)	46±6·3 (21)	47±8·2 (19)	
Difference . .	41·2±8·4	14·6±8·8	21·9±9·9	4·7±11·4	
Significance . .	ss	ns	s	ns	ss

The results are not conclusive, for apparently the temperature of 17° C. has exerted a devernalising effect, as is seen in the unheated controls. The 'score' in the unheated controls was reduced by 10·1±3·3 after 6 weeks of vernalisation and by 32·0±8·7 units after 4 weeks of vernalisation.

Assuming that the effect of the temperature of 17° C. on the subsequently heated grain was similar to that on the control, the scores for the heated set can be adjusted as shown in Table XII.

TABLE XII

Days' growth at 17° C. before heat treatment.	'Scores'	
	6 weeks vernalisation.	4 weeks vernalisation.
0	100	43
2	99	69
3	105	62
4	107	79

There is thus some evidence that devernalisation is reduced by growth made during a period at moderate temperature after vernalisation and before heat treatment is given. The seedlings vernalised for 4 weeks show this effect more strongly, as would be expected, since after 6 weeks vernalisation the condition is already largely stabilized.

A further experiment on the effect of the delay in application of heat treatment after the end of the vernalisation period was carried out in 1950. The germinating seed was as usual given 6 weeks' low temperature, then exposed to a temperature of 15° C. for 0, 2, and 4 days, before being heated for 3 days at 35° C. The results are entered in Table XIII.

TABLE XIII

Effect of Delayed Heat Treatment of Winter Rye Sown in Pots, April 28, 1950. Number of Replicates in brackets

Days' growth at 15° C. in sand after vernalisation for 5 weeks .	0	2	4
Length of coleoptile at time of heating .	0·4 cm.	1·1 cm.	2·4 cm.
Unheated 'score' .	111·2±1·5 (24)	111·4±1·2 (24)	115·1±0·63 (24)
Leaf Number . .	10·9±0·14	11·5±0·21	9·5±0·18
Heated 'score' . .	73·2±6·4 (21)	86·8±4·7 (24)	105·5±1·2 (22)
Leaf Number . .	16·0±0·78	14·3±0·76	11·3±0·26
Difference in 'score' .	38·0±6·4	24·6±5·4	9·6±1·4
Leaf Number difference	5·1±0·8	2·8±0·8	1·8±0·3

The results show that devernalisation by high temperature is much reduced by intercalating a period at 15° C. between the end of low temperature and subsequent heating. The results given in Table XI are thus confirmed, and in this case the devernalisation in the intervening period at 15° C. does not occur in the unheated set.

The large devernalising effect of a temperature as low as 17° C. as seen in Table XI seemed sufficiently striking to warrant confirmation, and in 1948 an experiment was carried out on winter rye vernalised for 4 weeks and then exposed for 3 days to temperatures varying from 15° C. to 40° C. before planting in pots. The results are shown in Table XIV.

TABLE XIV

Effect of Temperature Level on Reversal of Vernalisation. Three Days' Heat Treatment after 4 Weeks' Vernalisation. Winter Rye sown in Pots on April 30, 1948

Temperature level, ° C.	Control	15	20	25	30	35	40
'Scores' .	95±1·9	91±3·9	86±7·1	77±7·6	74±9·0	74±7·5	68±9·6
Significance of differences from controls .		ns	ns	$P = 0{\cdot}05$	$P = 0{\cdot}05$	$P = 0{\cdot}02$	$P = 0{\cdot}01$

Temperature as low as 15° C. has led to some devernalisation, and the effect increases as the temperature is raised, but in no case is complete reversal achieved.

VI. REVERNALISATION AFTER HEAT TREATMENT

To establish the reversibility of the vernalisation process in either direction, and to demonstrate that no permanent effect followed the heat treatment, the most direct test is to replace the heated seedlings at low temperature and to ascertain whether a second vernalisation can be carried out. The experiment was first tried in 1944. Seedlings were vernalised for 6 weeks and then exposed to high temperature for varying periods. They were then given a further 6 weeks of vernalisation. The 'Russian technique' was used. A second control series were vernalised continuously for 12 weeks. The results are entered in Table XV.

TABLE XV

Effect of 6 Weeks' Vernalisation following Heat Treatment at 35° C. on Seedlings originally vernalised for 6 Weeks. Sown in Field, May 1–5, 1944. Number of Replicates in brackets. Results in Conventional 'Scores'

Duration of heat .	0	8 hrs.	16 hrs.	1 day	2 days	3 days	4 days	5 days
Vernalised 6 weeks followed by heat .	100±3·0 (47)	95±4·6 (44)	90±4·9 (35)	75±6·5 (35)	80±5·9 (39)	72±9·5 (16)	72±6·4 (33)	63±66· (35)
Vernalised 6 weeks followed by heat and further 6 weeks of vernalisation . .	112±1·2 (17)	113±1·0 (48)	117±0·8 (41)	118±0·5 (42)	113±0·9 (39)	118±0·7 (54)	117±0·6 (54)	116±0·7 (55)
Vernalised 12 weeks . followed by heat	112±1·2 (17)	—	114±1·0 (40)	116±1·2 (75)	115±0·9 (42)	108±3·3 (41)	116±0·6 (55)	115±0·7 (57)

The data in the table show that a second period of 6 weeks of vernalisation after the heat treatment has completely revernalised the plants; indeed the 'scores' for the revernalised seedlings are in complete agreement with those of plants vernalised without a heat break for 12 weeks.

VII. DISCUSSION

The results presented in this paper provide conclusive evidence for the reversibility of vernalisation.

This has been shown in the following ways:

1. By interpolating during the low-temperature treatment a variable number of periods at 20° C. In this experiment (Table I) the *total* period at low temperature (1·8° C.) was kept constant at 6 weeks and the intercalated periods (in each case 24 hours at 20° C.) were given every alternate, third, fourth, and seventh day respectively.
2. In this experiment (Table II) the *total* times at low temperature and high temperature were always the same and in the ratio of 2:1, but the absolute durations were changed. The aim of this experiment was to test the change in sensitivity of the seeds to the temperature effects during the course of germination.

3. The effects of high temperature treatment (35° C.) of varying duration was studied immediately following vernalisation for 6 weeks at 1° C. Two methods of vernalisation were employed: first, on sand with unrestricted water-supply (Table III); and second, with restricted water-supply (Russian technique) to prevent excessive growth (Table IV). To test the effect of high temperature as such spring rye was grown simultaneously under the same conditions.

All these experiments establish the possibility of reversing vernalisation by high-temperature treatment, but the effects of giving the high temperature during the course of vernalisation and at the end of vernalisation must be clearly distinguished.

1. The results in Table I show that with increasing number of intercalated periods at 20° C. given during vernalisation the effects of a total period of 6 weeks at 1·8° C. are progressively annulled as the ratio of time spent at low and high temperature decreases. The early work of the authors (Gregory and Purvis, 1938) in which anaerobic conditions were maintained during the high temperature periods to prevent excessive growth is thus confirmed, and in the present work no anaerobiosis was imposed, but instead excessive growth was prevented by low water-supply (Russian technique). Anaerobic conditions in the earlier work therefore do not invalidate the conclusion that the vernalising process is reversible as Bassarskaya and Grossman (1941) have suggested.

2. When the relative duration at high and low temperature is maintained constant the devernalising effect is greatest when the *absolute* duration of the alternating periods is least (Table II). Alternative explanations of this effect are possible, thus: (*a*) the grain may be more sensitive to high temperature in the early stages of vernalisation; (*b*) the rate of vernalisation increases with time; (*c*) the reverse reaction due to high temperature increases with time. Each of these possibilities will now be examined.

(*a*) To test the sensitivity of the grain at varying stages of vernalisation a uniform heat treatment may be given at different times after the beginning of vernalisation. Results of such an experiment are shown in Table VI, in which 3 days at 35° C. were given after varying periods of vernalisation. It is clear that in early stages of vernalisation reversal is much more complete; indeed, in field culture the effect of 2 weeks' vernalisation is completely annulled by an exposure at 35° C., which, however, after 8 weeks reduces vernalisation by only 3 per cent. With increasing duration of vernalisation the reversal becomes progressively less. The vernalised condition thus becomes increasingly stable, and, as seen in Table X, after 12 weeks of vernalisation heat treatment at 35° C. has no effect even when given for 5 days, with which treatment a considerable proportion of grains are killed; the survivors are unaffected so far as vernalisation is concerned.

It may therefore be concluded that the grains are more sensitive to high temperature in the early stages of vernalisation.

(*b*) The 'duration' curve of vernalisation relating the 'score' to the period of vernalisation has been fully discussed by Purvis (1948), and there is little doubt that, using 'scores' as measure of intensity of vernalisation, the 'duration' curve is of the autocatalytic type, thus with a rising rate of vernalisation in the early stages to reach a maximum and then declining. The relation can be seen from the values in column 4 of Table VI.

(*c*) The changes of the rate of the reverse reactions will be considered immediately.

3. The effects of high temperature exposure of varying duration after 6 weeks of vernalisation (Tables III and IV) show that the relation of devernalisation with increasing duration of high temperature does not depart significantly from a linear relation, so that there is no evidence of any increase in sensitivity during 5 days' exposure to high temperature. The results obtained with winter rye in this work show that the degree of devernalisation depends upon the duration of the previous vernalisation period, and thus the discrepancy of this work with that of Efeikin and Tetjurev may be more apparent than real.

The control experiments carried out with spring rye indicate, first, that devernalisation of winter rye is not due to any deleterious effects of high temperature, and second, that no more devernalisation of spring rye can be accomplished by high temperature than was already obtained previously with anaerobic treatment (Studies, III).

Dynamics of the devernalisation reaction

The data in Table XIV show that the degree of devernalisation depends upon the level of temperature to which the partially vernalised grain is exposed. Some devernalisation occurs at 15° C. and at 17° C. (Table XI), and the effect increases towards a maximum up to the limit of 40° C. It has already been shown that vernalisation occurs up to a temperature approaching 10° C. It thus appears that between 10° C. and 15° C. a neutral temperature must exist at which no effect of temperature on subsequent flowering occurs.

The problem of estimating quantitatively the devernalising effect is by no means simple, and the mode of approach will depend upon the theory postulated. It has been assumed that devernalisation consists in a reversal of the vernalising reaction, so that the first stage in vernalisation is a reversible reaction. If, therefore, cycles of alternating high and low temperature are given during germination, in each low-temperature period advance towards the vernalised condition is made, and in the following high-temperature period, by reversal of the reaction, the effect is partially annulled. The effect of a single cycle will thus depend upon the temperature coefficients of the forward and backward reactions. The total effect of the series of cycles will depend upon the mode of summation of the effect of the individual cycles. Further, the total effect will depend upon the change in sensitivity of the grain as germination proceeds.

Let z be the 'score' of the vernalised but unheated grain, y be the 'score' of the vernalised and heated grain, n be the number of cycles given, and x the reduction of vernalising effect due to the high temperature in each cycle. Then the assumption is made that the following relation holds:

$$y = zx^n.$$

Applying this analysis to the data already presented, the values given in Table I for varying periods at low and high temperatures in a total exposure to low temperature of 6 weeks yield the following results.

TABLE XVI

Time at low temp. / Time at high temp.	'Score'.	No. of periods (n).	Log x.	x.	% reduction.
Control.	120	—	—	—	—
6:1	100	7	$\bar{1}$·9887	0·9745	2·5
3:1	85	14	1·9893	0·9756	2·4
2:1	64	21	$\bar{1}$·9870	0·9705	2·9
1:1	41	42	$\bar{1}$·9889	0·9748	2·5

The values in the last column represent the percentage reduction in the advances towards the vernalised condition in each period of exposure to high temperature. These values are seen to be nearly constant. This would indicate that the sensitivity to high temperature, or the temperature coefficient of the reverse reaction, remains constant during the whole period of vernalisation. Later work has, however, not confirmed this, but has shown variation in the sensitivity at different stages of vernalisation. This, indeed, would be expected from the fact that as vernalisation proceeds towards completion the degree of reversal by a given period at high temperature falls (Tables VI and X).

The same method of analysis was applied to the values in Table II, i.e. for an experiment in which the *total* periods at low temperature and high temperature were maintained at a ratio of 2:1, but the absolute durations of the cycles varied. The results are entered in Table XVII.

As each of the cycles in this experiment varied in the duration of the period at high temperature the percentage reduction per cycle and per day are entered in the table, and the latter figure alone can be compared with the values in Table XVI. The results for the two experiments in pot and field culture entered in Table XVII show a similarity in trend and are in general in fair agreement. The values for the ratio 2:1 agree remarkably well, but the value is almost double that in Table XVI. It thus appears that the reversing effect may vary from year to year, but that when grown in one season under such different conditions as in pots and in the field good agreement may be achieved.

TABLE XVII

Culture in Pots

Days at low temp. / Days at high temp. in each cycle.	'Score'.	No. of cycles.	Log x.	x.	% reduction per cycle.	% reduction per day.
Control.	107	—	—	—	—	—
42:21	111	1	—	—	—	—
21:10½	99	2	$\bar{1}$·9831	0·9618	3·8	0·4
14:7	67	3	$\bar{1}$·9322	0·8555	14·4	2·1
6:3	46	7	$\bar{1}$·9478	0·8867	11·3	3·8
2:1	35	21	$\bar{1}$·9769	0·9482	5·2	5·2

Culture in Field

Days at low temp. / Days at high temp. in each cycle.	'Score'.	No. of cycles.	Log x.	x.	% reduction per cycle.	% reduction per day.
Control.	87·5	—	—	—	—	—
42:21	100·2	1	—	—	—	—
21:10½	77·8	2	$\bar{1}$·9745	0·9428	5·7	0·6
14:7	42·2	3	$\bar{1}$·8945	0·7843	21·6	3·1
6:3	34·0	7	$\bar{1}$·9414	0·8736	12·6	4·2
2:1	29·5	21	$\bar{1}$·9775	0·9484	5·2	5·2

The results in Table XVII show that the longer the duration within a single cycle of the exposure to low temperature, the less is the reduction per day of exposure to high temperature. This merely confirms the previous findings: (1) that the longer the exposure to low temperature the more stable does the vernalised condition become; (2) that the rate of vernalisation increases in the early stages. As already pointed out, the values in Table XVI do not show this result.

The method of analysis is sufficiently promising to warrant further investigation. By using various temperatures during the high-temperature portion of the cycle it is hoped to establish the temperature coefficient of the devernalisation process by a comparison of the percentage reduction in the vernalising effect per cycle by the method indicated. This work is now in progress.

In the light of the results obtained in the experiments reported above the schema put forward by Purvis and Gregory (1937) requires reconsideration. This scheme is reproduced below.

$$\begin{tikzcd} & E & & \\ A \arrow[r] & B \arrow[u] \arrow[r, shift left] & C \arrow[l, shift left] \arrow[r] & D \end{tikzcd}$$

A represents the precursor from which a specific substance B is produced by a reaction at low temperature. The substance C is produced by a reversible reaction proceeding forward in darkness (short-day induction) and reversed in light. The reaction C—D proceeds only in light. C and D may be regarded as substances responsible for flower initiation and development, and these reactions occur in the terminal meristem. The substance B, alternatively, may be converted into the substance E, which is a vegetative hormone favouring leaf production.

The substance B is present in the embryo of spring rye and its formation occurs independently of temperature. Vernalisation of grain in the ear (Studies, II) has shown that the reaction $A \rightarrow B$ can proceed at low temperature during the formation and maturation of the embryo of winter rye.

From the work here reported it is clear that the reaction $A \rightarrow B$ consists of at least two stages; the first ($A \rightleftarrows A'$) is reversible by high temperature so that devernalisation and revernalisation are possible. The second stage, ($A' \rightarrow B$), however, can proceed at either normal or low temperatures. This is shown by the following facts: (1) as vernalisation proceeds the vernalised condition becomes more and more stable and irreversible by heat treatment, so that the reaction $A' \rightarrow B$ takes place at low temperature; (2) delay in application of heat treatment by maintaining the partially vernalised seedling at 17° C. (Table XIII) leads to a decrease in devernalisation by subsequent heat treatment, indicating that the reaction $A' \rightarrow B$ can also proceed rapidly at normal temperature. After heat treatment the 'score' rises from 73 to 105 by 4 days' exposure to 17° C. previous to heat treatment (Table XIII). To achieve a similar increase in 'score' after heat treatment given immediately after vernalisation requires 2 weeks approximately of extra vernalisation (Pot Culture, Table VI). The rate of the reaction at 17° C. is thus of the order of 3·5 times that at 1·8° C., giving a Q_{10} of 2·3. The scheme must therefore be amended as follows:

$$\begin{tikzcd} & & E & & \\ A \arrow[r, shift left] & A' \arrow[l, shift left] \arrow[r] & B \arrow[u] \arrow[r, shift left] & C \arrow[l, shift left] \arrow[r] & D. \end{tikzcd}$$

In spring rye the stage A' is entirely absent, and the reaction $A \rightarrow B$ is genetically controlled and is independent of temperature of germination. There is evidence of a slight reversal $B \rightarrow A$ by high temperature in spring rye similar to the partial devernalisation by anaerobic conditions during germination previously reported (Studies, III). The substance B in spring rye is evidently nearly thermostable, and a similar substance accumulates in the grain of winter rye as vernalisation proceeds. After 12 weeks' vernalisation the reactions $A \rightarrow A' \rightarrow B$ are complete and heat treatment then has no devernalising effect on winter rye (Table XIV).

SUMMARY

1. When Petkus winter rye is exposed to a temperature of 1–2° C. for a period totalling 6 weeks which is interrupted at regular and varying intervals by 1-day exposures at 20° C., the effect of low temperature in accelerating flowering is progressively annulled as the proportion of the total duration of the treatment spent at the higher temperature increases, i.e. as the ratio

$$\frac{\text{time at high temperature}}{\text{time at low temperature}} \text{ is increased.}$$

2. When the relative durations of exposure to low and high temperature are maintained at a fixed ratio (2 : 1) the degree of reversal diminishes as the *absolute* length of the cycle increases, indicating that sensitivity to reversal diminishes as vernalisation proceeds.

3. This is substantiated by experiments in which heat treatment (3 days at 35° C.) is given after vernalisation for different periods. The degree of devernalisation by heat varies inversely with the duration of the previous vernalisation treatment. After 12 weeks' vernalisation no devernalisation occurs.

4. The vernalised condition is further stabilized if a period of growth at 15° C. immediately precedes the heat treatment.

5. The increased leaf number and tillering after heat treatment shows that a true reversal of vernalisation is concerned rather than an adverse effect on general growth of the plants.

6. Some devernalisation follows exposure to temperatures as low as 15–17° C.: its magnitude varies with the temperature level and duration of exposure.

7. After heat devernalisation further exposure to low temperature again vernalises the grain completely, thus establishing the reversibility of vernalisation in either direction.

8. Petkus spring rye is very little retarded by heat treatment.

9. A method of assessing quantitatively the rate of the devernalisation process is described.

10. A revised scheme of the whole vernalisation process is presented.

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Chromosome Number of *Macrocystis integrifolia* Bory

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With Plate I and one Figure in the Text

ABSTRACT

Cultures of *Macrocystis integrifolia* zoospores have been made in sea-water enriched with phosphate and nitrate. It has been shown that the spores and gametophytes possess nuclei with the haploid complement of 16 chromosomes while the sporophyte nuclei are diploid with 32 chromosomes. Spores were transported by air from British Columbia to the British Isles. Results are compared with other Laminariaceae.

MACROCYSTIS integrifolia Bory and *M. pyrifera* Ag. are members of the Laminariales (Lessoniaceae) and the largest of the perennial algae. The distribution of *Macrocystis* is antarctic circumpolar, although also well represented on the western shores of North America. *Macrocystis* usually grows in deep water, 8 to 10 fathoms (Fritsch, 1945), but *M. integrifolia* can grow at L.W.M. providing suitable stable substrata are available (Scagel, 1947).

Above the holdfast of *Macrocystis* follows a long slender stipe which measures up to 60 ft. in exceptional cases (Scagel, loc. cit.) and bears a number of lanceolate blades constituting the greater part of the thallus which floats horizontally on the surface of the sea by the presence of an air-bladder at the base of each blade. More than one such stipe can arise from the same holdfast.

The sori of *Macrocystis* are usually formed on the blades of short shoots arising at the base of the plant. The sporangia liberate zoospores which are motile, using two laterally inserted flagella as means of locomotion. After a short period the zoospores settle, lose their flagella, produce a germ-tube, and develop into a male or female gametophyte (Pl. I, ABC). These gametophytes are microscopic multicellular filamentous plants and can branch extensively. The cells of the male plant are smaller than those of the female.

The gametophytes produce respectively spermatozoids and ova. The spermatozoid is motile and fertilizes the ovum in the oogonium which remains attached to the female gametophyte for a time. The resulting zygote from the fusion of the egg and sperm develops into an embryo sporophyte (Pl. I, D). Cell division and differentiation ultimately produce the mature, macroscopic sporophyte.

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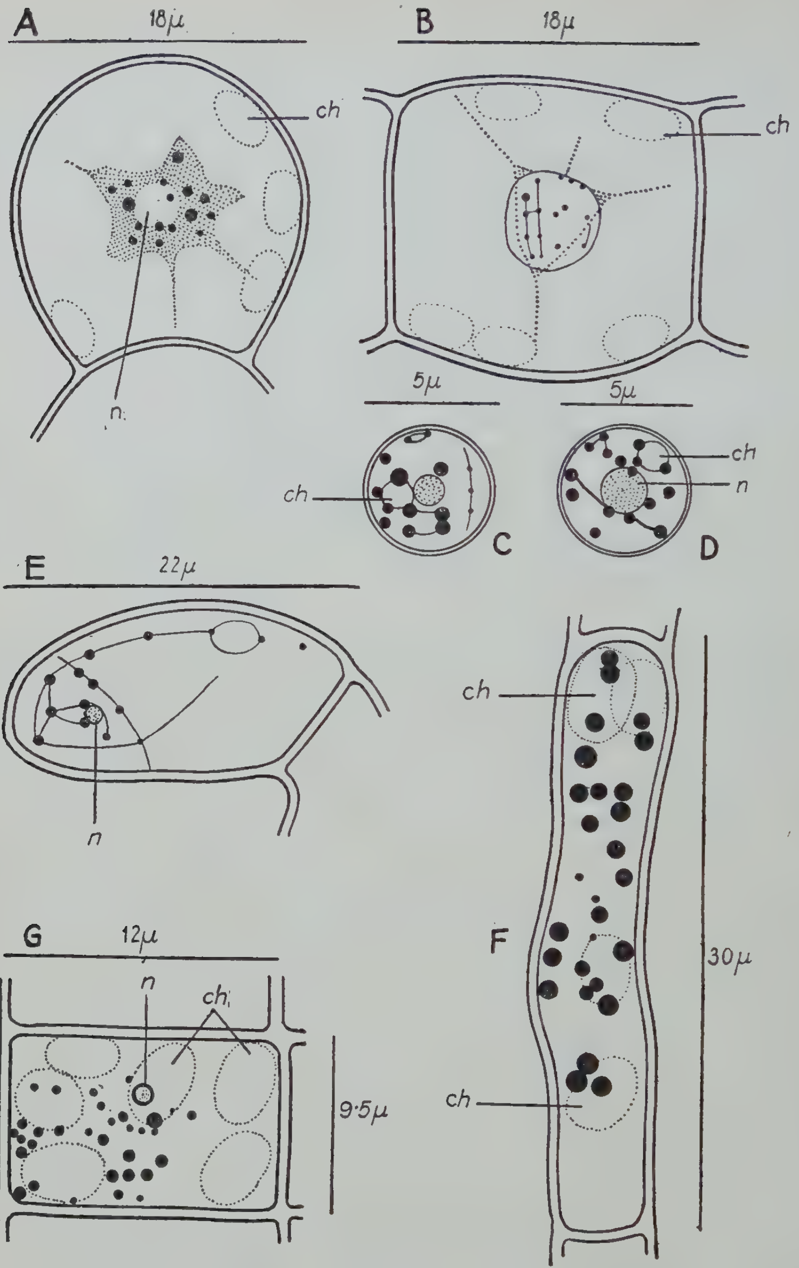

TEXT-FIG. *Macrocystis integrifolia.* A. Terminal cell of female gametophyte. Nucleus breaking down. 16 chromosomes and nucleolus seen. (×2,200.) B. Cell of female gametophyte. Nucleus intact. 16 Chromosomes seen, some still connected by threads. (×2,200.) C. Spore showing 15 chromosomes and nucleolus. (×3,400.) D. Spore showing 16 chromosomes and nucleolus. (×3,400.) E. Terminal cell of female gametophyte. 15 chromosomes seen dispersed in the cytoplasm but connected by threads; small nucleolus present. (×2,100.) F. Cell of male gametophyte dividing. 28 chromosomes are shown. (×2,600.) G. Marginal cell of young sporophyte. 32 chromosomes dispersed in the cytoplasm; nucleolus present. (×3,000.)

The chromosomes vary in size in each cell.

Sauvageau (1915) discovered the heteromorphic alternation of generations with two sexes of gametophytes in *Saccorhiza bulbosa* and later in *Laminaria digitata* and *L. saccharina*. Delf and Levyn (1926) added *Macrocystis pyrifera* to the list of Laminariaceae undergoing the same alternation. *Macrocystis integrifolia* has been found to follow the same cycle of development.

The plants used in this study were cultured from zoospores of *Macrocystis integrifolia* collected offshore from British Columbia and transported in sea-water by air to Prestwick, Scotland, during August 1949. From the Pacific to the culture tanks less than 2 days elapsed.

The culture solution was sea-water from the Firth of Forth enriched with additional phosphate and nitrate and well aerated.

Young sporophytes appeared in 36 days, which compared with 30 days for *Laminaria cloustoni* in similar culture solutions.

Cytological studies of the spores, gametophytes, and sporophytes of *Macrocystis integrifolia* show the basic chromosome number to be $n = 16$. The spores and gametophytes possess nuclei with the haploid complement of chromosomes, namely 16, while the sporophyte nuclei are diploid with 32 chromosomes (Text-fig. A–G).

In preparing for division the nuclear wall breaks down and the chromosomes and nucleoplasm are dispersed in the cytoplasm wherein chromosome division occurs.

The individual chromosomes are spherical and of varying size, but during certain phases they are connected by fine threads. The size of the smaller chromosomes and connecting threads are near the limit of resolution of a good microscope.

Usually a nucleolus can be seen which varies considerably in volume at various stages of the cytological cycle.

The chromosome number of $2n = 32$ for *Macrocystis integrifolia* is not the same as that for *Laminaria cloustoni*, where $2n = 22$ (Walker, unpublished).

Perhaps the most interesting phase of this investigation has been the successful transport and culture of spores from the other side of the world, for it opens up new fields for the algologist and the cytologist.

ACKNOWLEDGEMENTS

I gratefully acknowledge the co-operation given by the Provincial Fisheries Dept., British Columbia; The Scripps Institute of Oceanography, La Jolla; Nova Scotia Research Foundation, Halifax; Dept. of Biology and Botany, Univ. of British Columbia.

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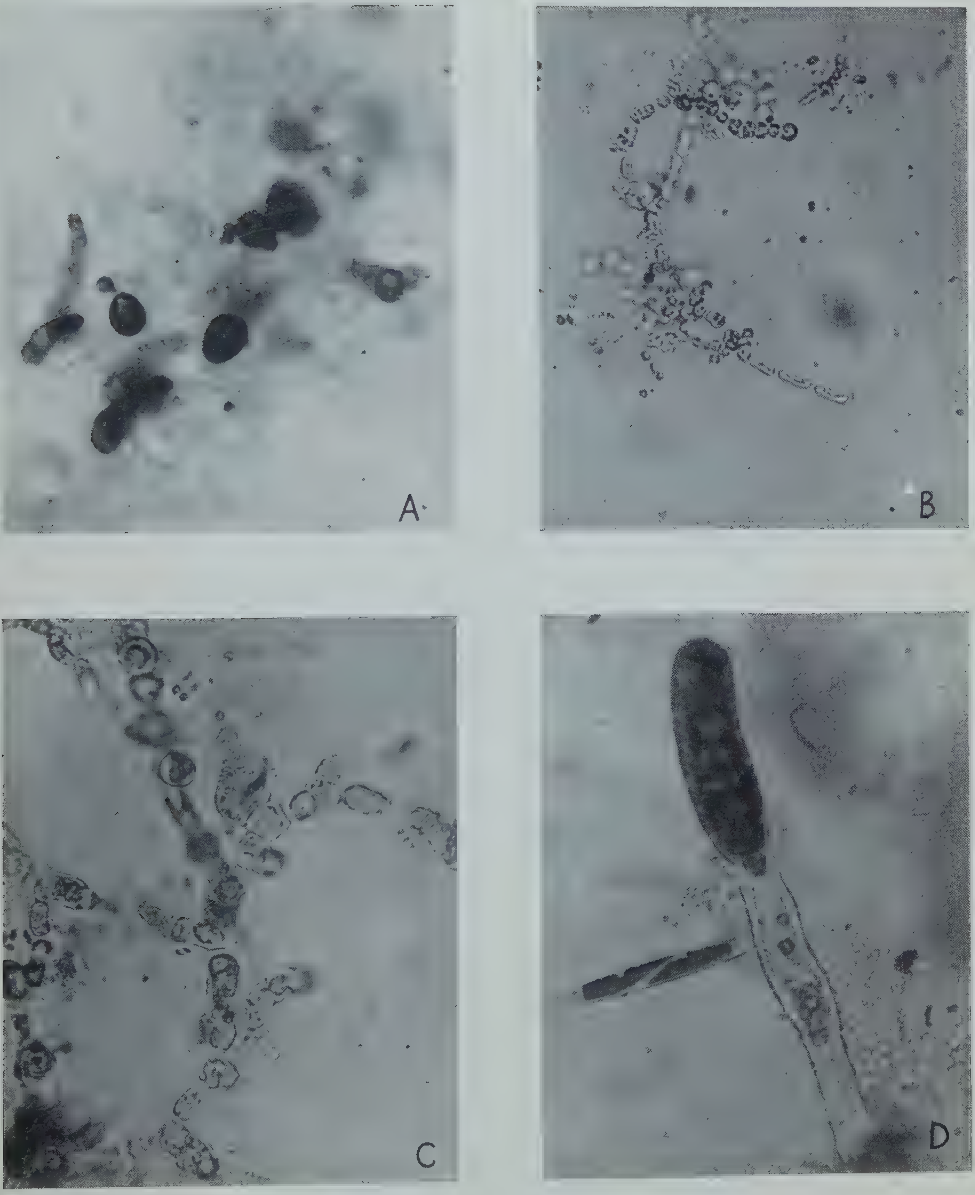
EXPLANATION OF PLATE

Macrocystis integrifolia

A. Early gametophytes. One plant seen dumb-bell stage. (×450.)
B. Male gametophyte. (×320.)
C. Female gametophyte. (×320.)
D. Embryo sporophyte, 8 cells. (×450.)

All plants photographed in the living state in sea-water. A and D were photographed with a blue-light filter.



F. T. WALKER

The Ovule and 'Seed' of *Araucaria Bidwilli* with Discussion of the Taxonomy of the Genus

II. Taxonomy

BY

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With Plates II and III and two Figures in the Text

ABSTRACT

This paper deals with the taxonomic and historical background of the genus *Araucaria* with a discussion and evaluation of characters used as a basis for sectional division. The three existing sections are more clearly delimited and a new section—Sectio *Bunya*—is proposed for *A. Bidwilli*, which is removed from section *Columbea*. A key to the four sections is given.

INTRODUCTION

IT has been apparent for some time that *A. Bidwilli* does not well fit into either of the two early established sections of the genus *Araucaria* nor does it fall into the recently established section *Intermedia* (White, 1947). Although its large leaves, axillary male cones, and hypogeal germination are characters similar to those of the two South American species of the section *Columbea*, its broadly winged cone-scales and geographical distribution (Queensland, Australia) separate it from the American species. Furthermore, it has been pointed out in a preceding paper (Wilde and Eames, 1948) that *A. Bidwilli* is unique in the genus in two important characters: the separate origin of the vascular supplies of the bract and ovuliferous scale and the shedding of the unusually large 'seed' from the bract-scale unit, a condition unlike that of all other known species in the genus. Part I of this study includes the morphology of the ovule, 'seed', and scale of *A. Bidwilli* as a basis for a modification of the sectional treatment of the genus. Part II presents the historical background of the present sectional treatment with a discussion of the relative value of sectional characters in the genus. Finally, a new section is proposed for *A. Bidwilli.*

HISTORICAL BACKGROUND

Towards the end of the eighteenth century the remarkable trees later known as araucarians were discovered almost at the same time in the eastern

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and western hemispheres. Sir Joseph Banks, the famous naturalist who sailed with Captain Cook on his first voyage round the world, brought back to England a specimen of a tree, later known as the Brazil pine, which he had collected in the vicinity of Rio de Janeiro as early as 1769.[1] With the exception of the Spanish settlers in that region he must have been the first European to see these trees. Description of this specimen, however, seems not to have been published, and the tree was later confused with the 'Chile pine'.[2]

In Chile, Don Francisco Dendariarena[3] in 1780 also found trees of this group in his search for timber suitable for the building of ships. Later Pavon saw great araucarian forests in the Cordilleras of Chile and found the Araucano Indians living upon the nuts. Molina (1782, p. 182)[4] published the first description of this tree in his 'Saggio sulla Storia naturale del Chile' under the name *Pinus araucana*. In the second edition of his history, however, he recounted that he had been well aware of the differences between the Chile pine and other members of the genus *Pinus* and that, therefore, in the original manuscript of his work he had given it the new generic name, *Pehuenia*[5] based on 'Pehuen', the native name for the tree. In the publication of his work, however, he had included it in *Pinus*, he said, because he had not wished 'to multiply' the many genera of plants in Chile. Dombey, who had accompanied Ruiz and Pavon on part of their travels in 1777 and later returned to Paris, gave specimens to both Lamarck and Jussieu. A description by Pavon apparently accompanied the specimens, for Pavon later wrote that neither of these botanists had made use of his information.[6] Lamarck (1786, p. 301) described the new plant as *Dombeya chilensis*, an unfortunate choice of name, as *Dombeya* had already been used for a sterculiaceous genus. Jussieu (1789, p. 413) established a new genus, based on specimens of the Chile pine, to which he gave the name *Araucaria*, apparently in accordance with Pavon's suggestion. Not until 1797 did Pavon publish his description of the species, calling it *Araucaria imbricata*. Until recent years the Chile pine has been commonly known under this name, although Koch (1873, p. 206) finally restored to it Molina's specific name *araucana* under the genus *Araucaria*.

While European explorers were penetrating into the New World, the naturalists of Captain Cook's second expedition in the famous sailing-ship

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[1] Salisbury (1807, p. 317), Bertoloni (1819, p. 411), Raddi (1827, p. 186), Parlatore (1861, p. 87).

[2] Lambert (1824, [p. 12]; 1828, vol. ii, p. 79). (According to Renkema and Ardagh (1930), the various copies of apparently the same issue of Lambert's great work often differ in content and arrangement. In this paper, all page numbers given in brackets refer, in the individual copies used, to pages numbered in pencil. Citations to the 1803, 1824, and 1832 ('editio minor') editions refer to copies in the Library of the Arnold Arboretum; citations to the 1828 and 1837 editions refer to copies in the Cornell University Library.) The authors are most grateful for the valuable assistance of Mrs. Lazella Schwarten, Librarian of the Arnold Arboretum, in the solution of some of the problems connected with Lambert's work.

[3] Lambert (1828, vol. ii, p. 77); Loudon (1844, vol. iv, p. 2436).

[4] American edition, Middletown, Conn. 1808 (vol. i, pp. 126, 247); English edition, London, 1809 (vol. i, pp. 148, 291).

[5] Molina (1810, p. 167); Raddi (1827, p. 187).

[6] Pavon (1797, p. 197). Translated in Lambert (1828, vol. ii, p. 77).

Resolution were finding in 1774 that the 'pillars of basaltes' on the shores of New Caledonia and adjacent islands were living trees of tall, columnar habit.[1] Specimens were brought back to England and described first by George Forster (1786, p. 67) under the name *Cupressus columnaris*. The description, however, was quite inadequate, dealing only with the leaves and the shape of the strobilus. At that time Forster believed trees that they had seen also on Norfolk Island to be of the same species as those on New Caledonia—a mistake that was not corrected for some years, and was likewise made by Lambert (1803, p. 87). In the rare and beautiful first edition of 'A Description of the Genus Pinus' Lambert published under the name *Dombeya excelsa*, 'Norfolk Island Pine', an equally inadequate description consisting of a few lines concerning leaf characters, and cited Forster's *Cupressus columnaris* as a synonym. But the specimens illustrated were from both New Caledonia and Norfolk Island;[2] those illustrated in the second edition of his work (1828), revised by D. Don, were under the name *Araucaria excelsa*. Since the source of each specimen illustrated in the coloured figures was carefully given, their identity is easily determinable. Although some doubts were expressed at that time[3] that the specimens from these two islands belonged to one species, it was not until 1813 that Robert Brown recognized that two species were involved. William Aiton, from a manuscript of Robert Brown, published in the second edition of his 'Hortus Kewensis' (1813, vol. v, p. 412) the name *Araucaria excelsa*, restricting the distribution of this species to Norfolk Island. He cited Lambert's *Dombeya excelsa* as a synonym and used Lambert's short description.

Robert Brown, moreover, seems to have been the first to include the two very different species known at that time, one from the eastern and one from the western hemisphere, in Jussieu's genus *Araucaria*. The New Caledonian species, Forster's *Cupressus columnaris*, Brown later, in 1841, named *Araucaria Cookii*,[4] without, however, furnishing further description. But as a distinct species of *Araucaria* this conifer was not mentioned in botanical literature for some time. Not until 1852 was Forster's specific name *columnaris* restored to it by Hooker,[5] who gave it at this time a complete description. Unfortunately the specific name *Cookii* has persisted in general use.

The present-day sectional treatment of the genus has its historical beginnings in the well-marked contrasting characters of these first-known species.

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[1] Forster, John Reinhold (1778, p. 174) (father of George Forster; both men sailed with Cook); Cook (1779, vol. ii, p. 133); Jackson (1865, p. 418).

[2] Lambert (1803, vol. i, Tab. 39, 40; 1828, vol. ii, Tab. 47). The description and figure of *Dombeya excelsa* were published in the appendix to the first edition of 1803. Renkema and Ardagh (1930, p. 442) cite evidence to show that the appendix was published in 1807. This is undoubtedly true, since Lambert alludes to Robert Brown's trip with Captain Mathew Flinders to Norfolk Island and the east coast of New Holland in 1804–5 and also to a painting of the Bay of Pines by William Westall in the 'Exhibition of 1805'. Furthermore, the figure of *Dombeya excelsa* is from a drawing by Ferdinand Bauer (Renkema and Ardagh, 1930, p. 442), a celebrated Austrian plant illustrator and collector, who with Robert Brown visited Norfolk Island in 1804–5 on Flinders' voyage.

[3] Lambert (1803, p. 89); Salisbury (1807, p. 317).

[4] Don (1841, p. 164).

[5] Hooker (1852, Tab. 4635).

These differences led Salisbury (1807, pp. 316–17) to place the species in new separate genera which he called *Eutassa* and *Columbea.*[1] The Chile pine he named *Columbea quadrifaria*; the Norfolk Island pine *Eutassa heterophylla.* The contrasting characters he used as a basis for separation were those of cone-scale, cotyledon number, and leaves.

For both groups he adds the phrase 'Flores terminales'. This character, true for the Norfolk Island pine, is inaccurate for the position of male cones in the Chile pine. These cones are axillary and solitary.

The Brazil pine, as represented by Banks' early specimen, seems for many years to have been confused with *A. araucana* from Chile.[2] Vellozo, a Spanish botanist who had spent twenty years in collecting, describing, and illustrating the flora of Brazil, finished the manuscript of this great work, 'Flora Fluminensis', in 1790 (Vellozo, 1881, p. x). In this flora he described and depicted the Brazil pine as *Pinus dioica.* But the text of his work was not published until 1825, and the eleven volumes of plates bear the date 1827.[3] Before Vellozo's work was published, specimens of the same tree were collected in 1818 by Giuseppi Raddi and a description published in the following year by Bertoloni (1819, p. 411), an Italian botanist, under the name of *Columbea angustifolia.*[4] Richard (1822, p. 512), apparently ignorant of Bertoloni's description, pointed out the differences between this Brazilian tree and the Chile pine and gave it the name *Araucaria brasiliana* (not *brasiliensis*). Raddi (1827, p. 187), in a paper read in 1824, called it *A. brasiliensis.*[5] In England Lambert (1824, p. 12, Tab. 5) described and illustrated it under the name *Araucaria brasiliana.* He seems to have been ignorant of the several earlier descriptions, since he did not cite any of them. Finally, Kuntz (1898, vol. iii, p. 375) restored to the Brazil pine the specific name *angustifolia* under the genus *Araucaria.*

From the eastern hemisphere the Moreton Bay pine, *A. Cunninghamii*, was not recognized as a distinct species until many years after its discovery. It was probably first seen along the coast of New Holland (Australia) by Cook, Banks, and Solander in 1770.[1] Robert Brown, who visited both Norfolk Island and the east coast of New Holland in 1804–5 on Captain Flinders' voyage, at first believed the pines of these two places to be of the same species as those of New Caledonia.[2] Later, however, when Brown recognized the Norfolk Island pine as distinct from the New Caledonian species (Aiton, 1813), he must have suspected the pine he had seen on the coast of Australia to be a different species, because he eliminated not only New Caledonia but New Holland as regions where the Norfolk Island pine is indigenous. Confirmation of his suspicion was apparently delayed at that time by his inability to obtain cones of the Moreton Bay pine. This uncertainty remained until 1824, when the explorer and botanist Allan Cunningham visited Moreton Bay, determined the species as distinct, and obtained fruiting specimens which were brought back to Kew.[3] The new species was given the name *Araucaria Cunninghamii* by William Aiton, then Director of Kew, and the name listed without description in 'Sweet's Hortus Britannicus' (1830, p. 475). In the same year it was listed by George Don as *Altingia*[4] *Cunninghami* in 'Loudon's Hortus Britannicus' (1830, p. 403), also without description. In the third edition (1839, p. 623) of the former work Don listed it as *Eutassa Cunninghamii*, restricting the genus *Araucaria* to the two South American species—in this sense synonymous with Salisbury's genus *Columbea*. The species was, apparently, undescribed until Lambert's description in 1837 (1837, p. 27). The proper designation of this species is, therefore, *A. Cunninghamii* Ait. ex Lamb.

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[1] Of 'Columbea' he writes: 'Nomen a voce graeca *χολυμβαειγ* nato: ob materiam navium malis peridoneam.'

[2] Parlatore (1861, p. 87).

[3] Vellozo, 1827 (*Pinus dioica*, vol. x, Tab. 55, 56). Only part of the text was published in 1825; an edition of the complete text was published in 1881 in the Archivos do Museu National do Rio de Janeiro (*Pinus dioica*, p. 408). Antonio da Arrabida, whose name is sometimes cited as authority for this binomial (Gordon, 1858, p. 23; Carrière, 1867, p. 596; and others), was the editor of the 1825 and 1827 editions.

[4] In a recent publication (Stellfeld, 1944, p. 181) the view has been stated that it would be 'more expressive' if we could use Vellozo's specific name—*Araucaria dioica*—for *A. angustifolia.* Under the rules of priority this would be quite out of order, since actual publication of Vellozo's work did not take place until 1825. Further, 'dioica' would not be more accurately 'expressive' since most and probably all species of *Araucaria* are monoecious. The genus is usually described incorrectly as dioecious.

[5] This species has been referred to in literature most frequently under the name *A. brasiliensis*, but with Richard cited as authority. The spelling *brasiliensis* seems to have arisen as an error (made perhaps for the first time) on a single plate by Lambert (1828, vol. ii, unnumbered plate, showing habit of tree, opposite p. 79) rather than as a citation by Lambert of Raddi's specific name, since Raddi's paper (in Italian) was published in a little-known Italian periodical and only rarely cited. Lambert had spelt the name correctly in 1824. The name *A. brasiliensis* has also unfortunately been widely used in horticulture.

In 1841 Link also attempted to limit the genus *Araucaria* to the two South American species. He placed *A. excelsa* and *A. Cunninghamii* in a new genus *Eutacta*, synonymous with Salisbury's *Eutassa*.[5] Contrasting leaf characters formed the primary basis of separation, although other characters were described, such as number of pollen sacs in *Araucaria* (not given for *Eutacta*), position of female cones, and cotyledon number.

In 1842 the first account of *A. Bidwilli* was published by its discoverer John Bidwill.[6] The botanical description of this species was made by Hooker in 1843 (p. 498).

[1] Lambert (1837, [pt. 2, p. 27]).

[2] Brown (1866, p. 48); Lambert (1803, p. 89).

[3] Lambert (1832, vol. ii, p. 115 and article 'Notes on the Moreton Bay Pine' by Allan Cunningham); Lambert (1837, vol. iii, pt. 2, p. 27); Jackson (1865, p. 420).

[4] Don had apparently followed Sprengel (1826, vol. iii, p. 888) in mistaking Noronha's *Altingia excelsa* (Hamamelidaceae) for the Norfolk Island pine and therefore placed the Moreton Bay pine in the same genus.

[5] Link used the name *Eutacta* from the Greek word εὔτακτος meaning 'well arranged'. Salisbury's *Eutassa* is compounded from the two Greek words εὖ (well) and τάσσειν (to arrange) (Wittstein, G. C., 1852: Etymologisch-botanisches Handwörterbuch). Link's name is apparently a good Greek word, whereas Salisbury's compound is certainly not good Greek. This seems to be Link's reason for using a new name for an already established genus. His own comment in Linnaea, xv. 544, 1841, is as follows: 'Eutactam vocavi, quam non graece clar. Salisbury Eutassam: nomen aptum ob regularem et bene ordinatum situm ramorum.' He rejected also the use of the generic name *Altingia* (Loudon, 1830) because it had been applied to another genus of plants by Noronha as well as to the Norfolk Island and Moreton Bay pines.

[6] Bidwill, H., 1842: 'On a New Species of Araucaria from New South Wales', Ann. Nat.

The first suggestions for division of the genus *Araucaria* (in its unrestricted sense) came from David Don (1841, p. 164)[1] in which Don pointed out that *A. excelsa*, *A. Cunninghamii*, and '*A Cookii* Br.' (*A. columnaris*) formed a related group within the genus, differing in certain characters from the South American species. For this group of species he suggested the use of Salisbury's old generic name *Eutassa*. The first actual division of the genus *Araucaria* in its unrestricted sense was made by Endlicher (1842, p. 26). In the second supplement of his 'Genera Plantarum' under the genus name *Araucaria* he listed '*a*-Colymbea, *b*-Eutacta'.[2] He did not specify the rank of these groups but designated them by '*a*' and '*b*'.

A division of the genus with its then known six species into subgenera was made by Antoine, probably about 1845 or 1846.[3] In 1847 Endlicher's 'Synopsis Coniferarum' was published. Without doubt Antoine's treatment of *Araucaria* appeared before Endlicher's 'Synopsis', since Endlicher cites Antoine's publication. For his subgenera Antoine used Link's generic name *Eutacta*, and, apparently following Endlicher's earlier work (1842), also mis-spelt Salisbury's *Columbea*. Endlicher, in his 'Synopsis', as in his 'Genera Plantarum', divided the genus into two groups without specifying taxonomic rank, indicating the groups merely by roman numerals—'I. Colymbea; II. Eutacta'. Antoine similarly used these roman numerals, but for his subgenera. Endlicher did not specify the rank of these groups, merely using the roman numerals I and II. Because of the frequent use of these numerals in this way at that time and later, it can be assumed that he intended them as sectional designations. He apparently intended to use Salisbury's name *Columbea*, but mis-spelt it '*Colymbea*' (he had earlier cited it correctly in his 'Prodromus Florae Norfolkicae', 1833); and he obviously preferred Link's generic name *Eutacta* (published the year before) to Salisbury's *Eutassa*.

Endlicher's treatment of the genus in his 'Synopsis' is strikingly similar to that of Antoine's. The similarity lies in the choice and order of new characters in addition to those he had used in his earlier treatment, and in some of the Latin phrasing, although he did not cite Antoine's work in this connexion. Both authors described the following contrasting characters in the same order:

Hist. viii. 438–9. The initial 'H' is obviously an error, as Bidwill is listed in the Dictionary of National Biography (London, 1886) and elsewhere as 'John Carne Bidwill. (1815–1853).' He is unfortunately variously cited as 'J. T.' and 'J. S.' Bidwill.

[1] The article is of further interest in that Don here proposed the group Araucarineae 'to consist of Araucaria, Dammara, and perhaps Cunninghamia' and to be coordinate with Richard's (1826) groups Abietineae, Cupressineae, and Taxineae.

[2] *a*-Colymbea—Cotyledones 2, lineares, semi-cylindricae, germinatione hypogeae—species americanae.

b-Eutacta—Cotyledones 4, germinatione epigeae—species australasecae.

[3] Antoine, F., Die Coniferen nach Lambert, Loudon und Anderen. The complete work of eleven parts, originally published separately, is bound in one volume which bears on its title-page 'Wien 1840'. The several parts were published over the period of years from 1840 to 1847, but it is apparently impossible to determine exactly the date of publication of each part. The part containing the treatment of *Araucaria* was apparently the second before the last.

Eutacta	*Colymbea*
Scales broadly winged	Scales obscurely winged
'Appendix', 'Squamula' obscure[1]	'Appendix', 'Squamula' visible
Chambers of the anther 6–12	Chambers of the anther 12–20
Cotyledons 4	Cotyledons 2–4
Germination epigeal	Germination hypogeal
Leaves of young plants heteromorphic (Endlicher, not Antoine)	Leaves of young plants homomorphic
Species Australian	Species American

Endlicher did not list species under his divisions of the genus in 1842. But in his 'Synopsis Coniferarum' of 1847 he listed under 'Colymbea', '*A. brasiliensis*' and '*A. imbricata*'; under 'Eutacta', *A. Bidwilli*, *A. Cunninghamii*, and *A. excelsa*. Antoine's treatment of the species is similar, except that apparently he was in doubt about the position of *A. Bidwilli*, for he left this species unnumbered in his list of species under *Eutacta*. Antoine does not mention *A. columnaris* but Endlicher lists it under *Eutacta* as 'Species 6, *A. Cookii*', but separated from the other species by the subheading 'Species indescripta'.

Nearly 20 years passed before the discovery of more species. These were four from New Caledonia: *A. Rulei*, described by F. von Mueller;[2] *A. Muelleri*, first described as *Eutacta Meullerii* by Carrière (1866, p. 392); and *A. Balansae* and *A. montana*, described by Brongniart and Gris (1871, pp. 206, 215). Von Mueller, in his specimens and in his published description and figures of *A. Rulei*, had apparently included two distinct species. This was pointed out by Carrière (1866, p. 393; 1867, vol. ii, pp. 605, 607), who established a new species, *Eutacta Muellerii*, represented by von Mueller's figures and based upon specimens in the herbarium in the Museum of Paris. Brongniart and Gris (1871, p. 219) later redescribed and figured this species under the name of *Araucaria Muelleri*, citing as a synonym *Eutacta Muellerii* of Carrière. The binomial should, therefore, be written *A. Muelleri* (Carr.) Brongn. et Gris.

The taxonomic position given by authors to *A. Rulei* and *A. Muelleri* within the genus has varied because, although the cone-scales of these species are of the *Eutacta* type, their cones are larger and their leaves are longer, larger, and flatter than those of the species of this group. *A. Rulei* was included in the *Colymbea* section of the genus by Henkel and Hochstetter (1865), Parlatore (1868), Gordon (1875), Veitch (1881), and more recently by Wilson[3] and Wieland (1935). Carrière (1867) seems to have been the first to include it with the *Eutacta* species; more recently Seward and Ford (1906) and Pilger (1926) have placed it there. *A. Muelleri*, considered a member of the *Eutacta* group by Carrière (1867) and Pilger (1926), was listed as a member of *Colymbea* by Wilson[3] and as a doubtful member of *Colymbea* by Wieland (1935).

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[1] The greatest difference lies in the wording of the statements referring to the fertile scale. Antoine: *Eutacta*—'Strobili squamae late alatae, vertice intus squamula auctae.' *Colymbea*—'Strobili squamae obsolete alatae, vertice intus squamula nulla vel rudimentaria auctae.' Englicher: *Eutacta*—'Strobili squamae late alatae. Seminum appendix basilaris manifesta.' *Colymbea*—'Strobili squamae obsolete alatae. Seminum appendix basilaris obsoleta'.

[2] In Lindley (1861, p. 868).

[3] In White (1926, pp. 82–85).

The distribution of *Araucaria* was further extended by the later description of several species from New Guinea. Schumann in Schumann und Hollrung (1889, p. 11) described *A. Hunsteinii*, which, like *A. Bidwilli*, has the surprising combination of long flat leaves and broadly winged cone-scales. This species was considered by these authors to belong in the *Eutacta* section, but it has been placed in *Colymbea* by Pilger (1926, p. 265). Warburg (1900, vol. i, p. 187) described still another New Guinea species, *A. Schumanniana*, closely resembling *A. Hunsteinii*, and Lauterbach (1913, p. 48) described a third New Guinea species as *A. Klinkii*. Although Lauterbach assigned *A. Klinkii* to the section *Eutacta*, Pilger (1926, p. 265) has placed it in the section *Colymbea*. All these New Guinea species have long flat leaves, but cone-scales (Pl. II, Figs. 7, 10, 11) even more broadly winged than those of other species (Pl. II, Figs. 1–6, 8, 9) generally assigned to *Eutacta*. Recently White (1947, p. 260) has proposed a new section of the genus for these three New Guinea species—Sectio Intermedia. White states that, in addition to the *Eutacta* character of broadly winged cone-scales, the type of germination in these species is similar to that of species of *Eutacta*. This combination of broad, flat leaves with characters of *Eutacta* has made necessary the creation of this new section.

In 1877 Beccari (vol. i, fasc. ii, p. 180) described an *Araucaria* from New Guinea which he believed to be *A. Cunninghamii*. Warburg (1900, p. 187), however, considered Beccari's specimen sufficiently different from the Moreton Bay pine to constitute a separate species which he named *A. Beccarii*. Gibbs (1917, p. 83) accepted this view and pointed out that the cone-scales[1] were larger and had a more prominent fertile-scale tip than those of the Australian *A. Cunninghamii*. Lauterbach (1913, p. 51), on the other hand, described this tree as *A. Cunninghamii* var. *papuana*, a treatment followed by Pilger (1926, p. 265). The true Moreton Bay pine has been reported in New Guinea by von Mueller (1887, p. 121), Lauterbach (1913, p. 51), White (1947, p. 259), and others. This extends the distribution of the *Eutacta* species.

The treatment of the genus in the early years immediately following Antoine's and Endlicher's publications was somewhat variable. Endlicher's diagnosis, as given in the 'Synopsis Coniferarum', was usually followed, with the addition of several new characters. A sectional division of the genus, ascribed to Endlicher, gradually became established. Gordon (1858) used the section *Colymbea* (citing as of Salisbury, but Salisbury's spelling is *Columbea*) and *Eutacta* (citing Link). To Endlicher's characters he added three new ones:[2] size of female cone, position of female cone in *Eutacta*, and relative persistence of cone-scales.

Carrière, with French versatility, divided the genus into 'tribes' in 1855, into sections in 1857, and into subgenera in 1867. In the first two divisions he followed Endlicher without change, but the 1867 edition of the 'Traité Général des Conifères' is notable for its new treatment. Here Carrière used subgenera and cited both Antoine's and Endlicher's work. He placed, first, the clearly defined leaf characters of adult, rather than juvenile, plants; to Endlicher's characters he added those of Gordon: cone size, position of female cones in *Eutacta*, and relative persistence of cone-scales. He qualified the type of germination in each case by the phrase 'en général'.

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[1] The cone-scale as illustrated by Gibbs (1917, p. 84), if it is figured natural size (no size is stated), is considerably larger than the measurements given by Beccari for the cone-scale of his specimen.

[2] Gordon's character: 'Seeds, indistinctly attached at the base', seems to be a mistranslation of Endlicher's 'Seminum appendix basilaris obsoleta'. Gordon (1858, p. 21).

Parlatore (1868) used similar sectional division, but returned to Salisbury's spelling of *Columbea*. He added leaf characters of adult plants (placed last) to Endlicher's characters and cited the works of Salisbury, Link, Endlicher, and Carrière. Gordon, in the second edition of 'The Pinetum' (1875), likewise returned to Salisbury's spelling of *Columbea*.

Subsequent treatments of the genus have varied little, usually only in the relative importance placed upon the different characters, as indicated by the order in which they are listed. Rarely has cone position, as first stated by Gordon (1858) and Carrière (1867) for *Eutacta*, been used by later authorities. Seward and Ford (1906), however, are an exception. In their treatment of the genus they used male-cone position for both sections: axillary in *Colymbea*; terminal in *Eutacta*. To the group of characters commonly taken from Endlicher they added a new character, suggested by Thibout's work (1896, p. 106), that of arrangement of microsporophylls: verticillate in *Columbea*; spiral in *Eutacta*. They rejected the character of pollen-sac number as unreliable and discussed the value of leaf anatomy (Bertrand, 1874) as a sectional character, concluding that it, too, is unreliable. Post and Kuntze (1904, p. 41) published the names of two sections as 'Dombeya O.K.', and 'Eutassa O.K.', synonymous with Endlicher's sections. No description accompanied the terms.

Most authors have accepted the names *Colymbea* and *Eutacta*, citing Endlicher as the sectional authority; a few have returned to the original spelling of Salisbury, *Columbea*, notably Koch (1873), Bentham and Hooker (1883), Beissner (1891), Bailey (1914), and Wilson (in White, 1926). The question naturally arises as to why Antoine and Endlicher used *Colymbea* rather than *Columbea*. Evidence seems to support the fact that the spelling *Colymbea* was copied by Antoine from Endlicher and earlier authors.

In the taxonomic publications of the period from 1807, the date of Salisbury's work, to 1845 or 1846, the probable date of Antoine's, the majority of citations to Salisbury's genus *Columbea* are mis-spelt. Apparently the earliest error is in Sprengel's 'Linnaei Systema Vegetabilium' (1826, vol. iii, p. 888), where Sprengel cites this genus as 'Colymbea Salisb'. Since he was apparently the first to mis-spell Salisbury's generic name (definitely citing it as such) and, four years later (1830), as editor of 'Linnaei Genera Plantarum', changed the name to *Columbea*, as Salisbury had spelt it, the presumption is that he thereby acknowledged his first spelling to be an error. Loudon, doubtless following Sprengel, also mis-spelt Salisbury's name in at least two editions of both his 'Hortus Britannicus' (1830, 1832) and his 'Arboretum et Fruticetum' (1838, 1844). Endlicher, in his earlier 'Prodromus Florae Norfolkicae' (1833), had cited Salisbury's genus correctly and also cited Sprengel's mis-spelling *Colymbea*. But in his 'Genera Plantarum' (1836–40), 'Enchiridion Botanicum' (1841), and his 'Supplementum Secundum' of his 'Genera Plantarum' (1842), he cited Salisbury's genus *Colymbea*. He therefore cited Salisbury both correctly and incorrectly. It is difficult to understand Endlicher's changes in spelling. Sprengel, using Salisbury's name as a generic name, clearly corrected his (Sprengel's) earlier mis-spelling. Endlicher, using the name as a sectional name—after having once published it correctly—similarly mis-spelt it, perhaps because at that time it was so spelt by other authors who were following Sprengel's error. But in his publications between 1836 and 1842 he clearly erred by citing Salisbury's genus as *Colymbea*, apparently forgetting that he had cited it in 1833 as *Columbea*.

Two of the sources to which Antoine went are known from the title of his work: 'Die Coniferen nach Lambert, Loudon und Anderen.' Lambert did not refer to Salisbury's genus; but the error, probably copied by Loudon from an earlier source, has been repeated many times by later authors. Obviously Link's *Eutacta* was preferred to Salisbury's *Eutassa* (the generic name *Eutacta* was adopted without change). Certainly evidence points to the fact that both Endlicher and Antoine intended to use Salisbury's *Columbea* as a sectional name and that the term *Colymbea* arose as an orthographic error, was copied and recopied many times from the early years, following Antoine and Endlicher to the present day. The original spelling of this name—*Columbea*—should therefore be maintained, as by some authors today.

Discussion of Subgeneric and Sectional Characters

Vegetative type and cone position

In many of the coniferous genera, including *Araucaria*, specialization and reduction of the leaf and branch system has resulted in the formation of two natural, well-defined vegetative types: *leaf-reduction* type, that in which the tendency for leaf reduction has been strong and branch reduction weak; and *branch-reduction* type, that in which a relatively unreduced leaf has been retained but branching has been greatly reduced. The degree to which branch reduction has occurred in a genus can be determined by the position of the male cone (or cone cluster) and by the number and character of the bracts and reduced leaves at its base.

Leaf-reduction type. The adult leaves of the leaf-reduction type in *Araucaria* are relatively small, thick, often laterally flattened or keeled, sometimes tetragonal in cross-section, and generally incurved. The juvenile leaves are larger and less reduced, the young plants during their change to the adult type appearing 'heteromorphic'. Within the group bud-scales are lacking.

The male cones of this type are produced terminally on the branches (lateral cones having been lost), and are surrounded at the base by bracts which generally differ in shape, thickness, and often size from the vegetative leaves.

For example, the bracts at the base of the male cones in *A. Cunninghamii* and *A. columnaris* are somewhat longer, flatter, thinner, and straighter than the vegetative leaves. Those of *A. Muelleri*, as shown in the excellent illustrations by Brongniart and Gris (1871, Pl. 15), differ similarly from the leaves. These bracts, clustered at the base of the solitary cone, appear to have been retained from a once long fertile branch bearing numerous cones, each in the axil of a bract, as in *Podocarpus spicatus* (Wilde, 1944, Fig. 3). In the reduction of the fertile branch all cones have probably been lost with the exception of a single terminal one, but the bracts with shortened internodes persist at the base of the single cone. In a primitive conifer fertile branches would be produced both laterally and terminally on vegetative branches, as in *P. spicatus*. In this group reduced fertile branches, bearing a solitary cone with its basal bracts, are probably produced only terminally on the vegetative branches.

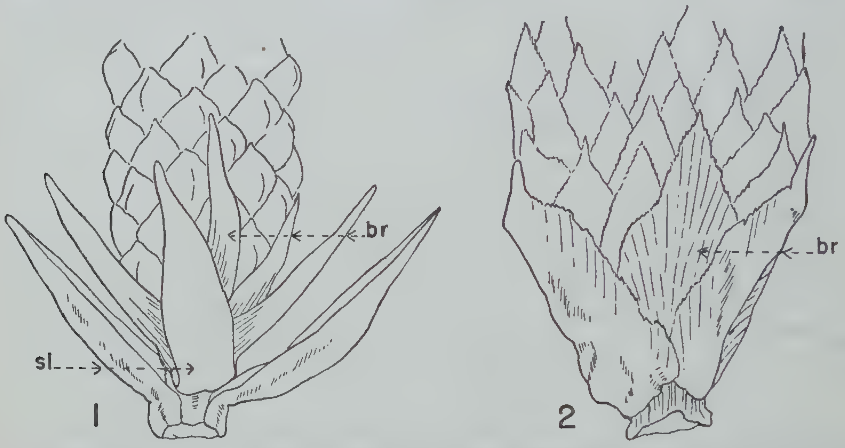
The female cones, like the male cones, are terminal and borne on stout elongate 'peduncles' (more or less leafy branches) several to many centimetres long.

Such a leaf-reduction type in *Araucaria* is clearly analogous to the type characteristic of the *Dacrycarpus* section of *Podocarpus* (Wilde, 1944, Figs. 39, 40), most species of *Dacrydium*, and probably most of the Cupressaceae. In *Araucaria* this type includes all those species commonly placed in the section *Eutacta*. *A. Rulei* and *A. Muelleri*, however, have been placed first in one, then in the other section of the genus, and their position still seems in doubt. The original descriptions of these species contain no statement concerning male-cone position. Brongniart and Gris (1871, Pl. 15), however, illustrate for *A. Muelleri* a male cone that appears to be terminal, and Dallimore and Jackson (1931) describe terminal cones for both species. Although these species have leaves that are thinner and longer than those typical of species of *Euctacta*, they resemble species of *Eutacta* in the position of the terminal male cone.

Branch-reduction type. The branch-reduction type in *Araucaria* is characterized by adult leaves longer, thinner, flatter, and more spreading than those in the leaf-reduction species, pointed at the apex, and usually markedly decurrent at the base, and by axillary male and female cones. Internodes are generally longer than those in species of *Eutacta*, but the size of the leaves may be variable.

In *A. Bidwilli* at the beginning of seasonal growth, as in many other conifers that have not attained specialized bud-scales, the leaves are smaller, although internodes have not been lost. Since there is no marked transition in leaf size from the juvenile to the adult state, leaves on juvenile plants are described as 'homomorphic'. That branch reduction, however, has taken place in this type is clearly shown by the axillary position and character of the peduncled male cone.

In *A. Bidwilli* the short, thick male peduncles usually bear two pairs of nearly opposite decussate leaves, above which at the base of the cones are two to several narrower spirally arranged bracts closely resembling the leaves (Text-fig. 1). The solitary male cone with its bracts probably represents the single surviving terminal unit of a fertile branch that was terminal on a now greatly reduced vegetative branch. Reduction of both vegetative and fertile branches has brought this single surviving cone down into an axillary position. Female cones in the branch-reduction species are axillary, sessile, or subsessile, with peduncles at most 2 cm. long. White (1947) describes the ovulate cone of *A. Bidwilli* as sessile, but in the authors' material, wild and cultivated, the peduncles are 0·5–1·5 cm. long. White is, of course, contrasting this with the greatly longer peduncles of Section *Eutacta*. That actually they are terminal on shortened lateral vegetative branches, as are the male cones, is more clearly evident in the young cones. The degree of shortening of this vegetative cone-bearing branch in the female has not, however, reached the extreme condition of reduction present in the male.



TEXT-FIGS. 1–2. Fig. 1. Base of male cone of *A. Bidwilli*, showing three of the four decussately arranged scale leaves (*sl*) surrounding several spirally arranged bracts (*br*). (×5.) FIG. 2. Base of male cone of *A. Klinkii*, showing three of the five spirally arranged bracts (*br*). No scale leaves are present as in the cone of *A. Bidwilli* (×5.)

A. Bidwilli and the two South American species, *A. araucana* and *A. angustifolia*, belong certainly to this type. The three New Guinea species: *A. Schumanniana*, *A. Hunsteinii*, and *A. Klinkii*, seem to resemble *A. Bidwilli* in vegetative type. These four species have been placed first in one, then in the other section of the genus. The New Guinea species, in their thin, broadly winged cone-scales, resemble species of *Eutacta*; in vegetative type, however, they have the large leaves and axillary cones of species of *Columbea*. For these three species White (1947, p. 260) proposed a new section, *Intermedia*, a treatment that appears justified.

During the course of this study the authors have been fortunate in securing male specimens and a mature female cone of *A. Klinkii* through the kindness of Mr. J. S. Womersley, Forest Botanist of New Guinea.[1] Since male cones of this species were not described or figured in the original description (Lauterbach, 1913), a photograph of a specimen bearing male cones is here included (Pl. III, Fig. 12). The male cones of *A. Klinkii* are borne singly, axillary to vegetative leaves, close together near the end of the vegetative branches. The photograph shows at the tip of the branch a new growth with young leaves partly hidden by the four cones which superficially appear like a terminal cluster. The large leaves, 8–10 cm. in length, and axillary male cones place *A. Klinkii* in the branch-reduction group.

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[1] Mr. Womersley has recently also made a search for the two other New Guinea species in the localities from which the types came, but has as yet been unable to find them. The authors would like to express their gratitude both to Mr. Womersley and to Mr. J. B. McAdam, Acting Secretary, Department of Forests, Territory of Papua, for their kind assistance in this matter.

A closer examination of the male cones, however, reveals *A. Klinkii* as more primitive in fertile-branch reduction than either *A. Bidwilli* or the two South American species. At the base of an axillary cone are four or five bracts, larger than the microsporophylls and arranged in a close spiral that approaches a condition of decussate pairs (Text-fig. 2). There is no trace of reduced vegetative leaves associated with the cones. Each single cone appears to represent the terminal unit of one shortened fertile branch that was borne laterally on a vegetative branch. In species of *Eutacta* the fertile branches that persisted were apparently terminal. Branch reduction seems to have involved only fertile branches in this species. This is a condition quite different from that found in *A. Bidwilli*, in which reduced scale leaves are found outside the bracts (Text-fig. 1), indicating that branch reduction in *A. Bidwilli* is more advanced than that in *A. Klinkii*, and that vegetative as well as fertile branches have been involved. A critical study of the other two New Guinea species is much to be desired. White (1947, p. 260), however, has expressed the opinion that 'it is possible that all three represent forms of one rather variable species'.

In defining the section *Intermedia* White (1947, p. 260) has not used the character of cone position; but has used characters of juvenile and adult leaves, seed, scale, and type of dissemination, with cotyledon number and type of germination.

Neither Antoine (1840–7) nor Endlicher (1847) in his division of the genus used the characters of mature leaf size or cone position. Link (1841), however, made use of leaf characters for generic segregation, while Carrière (1867) and Seward and Ford (1906) included both leaf characters and male-cone position in their treatments. The characters of vegetative type and cone (male and female) position (that is, axillary or terminal) appear to be excellent in separating the sections *Eutacta* and *Columbea*. The more primitive condition of branch reduction, with the presence of bracts alone at the base of the axillary male cones in *A. Klinkii*, further supports the new section *Intermedia*.

Cone size

Gordon's (1858) character of female-cone size, recently stressed by Wieland (1935, p. 117), does not appear reliable. In general the female cones of species of *Eutacta* are smaller than those of species of *Columbea*. In most species of *Eutacta* the female cones are about 8 to 10½ cm. in length, but those of *A. Muelleri* are considerably larger and approach the size of cones in species of *Columbea* which are about 13 or 14 cm. long. Female-cone size in the three

New Guinea species, as given in the few published descriptions, appears to vary considerably, and cannot, therefore, be used as a sectional character. *A. Bidwilli* has without doubt the largest female cones of any known species of *Araucaria*, reaching in cultivation a length of 30 cm. and a diameter of 28 cm. In this character it again stands apart.

Male cones of most species of *Eutacta* are, like the female cones, generally smaller than those of species of *Columbea*. In *A. Rulei* and *A. Muelleri*, however, the male cones are considerably larger than those in other species of *Eutacta*. In fact the male cones of *A. Muelleri* are apparently the longest in the genus. The leaves also of these two species are longer than those of other species of *Eutacta*. Mere size of leaves and cones, however, is not sufficient reason for including these species in *Columbea*. They seem to resemble species of *Eutacta* in the far more important characters of vegetative type, cone position, type of bract-scale unit, and germination. Again there is considerable variability in cone size among the New Guinea species. Although the male cones of *A. Bidwilli* are longer than those of species of *Columbea*, they fall within the range of those of the New Guinea species.

Microsporophylls and number of sporangia

According to both Antoine and Endlicher the number of microsporangia in species of *Eutacta* lies between 6 and 12, in *Columbea* between 12 and 20. This was possibly true of the species known to Antoine and Endlicher, but more than 12 may occur in *A. columnaris* and less than 12 in *A. angustifolia*, while *A. Rulei* and *A. Muelleri* with higher numbers again deviate from other species of *Eutacta*. As in cone size, there is wide variation in number of microsporangia in the three species of the section *Intermedia*. In general the smaller number of microsporangia is found in species of *Eutacta*, but the limits in microsporangial number for each section are not sufficiently sharp to render this character useful. From an examination of the male cones of *A. Bidwilli*, *A. araucana*, *A. columnaris*, and *A. Klinkii*, the character suggested by Seward and Ford (1906) of verticillate contrasted with spiral arrangement of microsporophylls appears likewise unreliable.

Type of germination

At the present time it is impossible to evaluate satisfactorily as group characters the type of germination and cotyledon number, since these characters are known for only a few species in the genus. Both Antoine and Endlicher made use of these characters and Pilger (1926) has emphasized their importance. They should be of morphological value, although their practical use for the taxonomist in the field is negligible. For those species investigated for this character, it has been found that germination in the *Eutacta* group is epigeal, that in *Columbea* hypogeal (Dürr, 1865; Guillochon, 1925, 1926; Hickel, 1911, 1926). Of the little-known New Guinea species, *A. Klinkii*, according to White (1947), is epigeal. Correlated with the differences in germination type in the sections are strong structural differences in early seedling morphology. In *Eutacta* the hypocotyl is slender; the sessile or subsessile cotyledons and the plumule are early freed from the seed-coats. In the South American species the hypocotyl is greatly swollen and fleshy; the cotyledons remain within the endosperm and the seed-coats, their bases elongating greatly (to several centimetres), much as do the cotyledon bases in *Quercus*, carrying the plumule exposed between them. In *A. Bidwilli* the hypocotyl is excessively swollen, forming a turnip-shaped tuber; the cotyledons remain within the endosperm and seed coats, their bases, fused into a hollow tube, elongating greatly and enclosing the plumule at the proximal end of the tuber (where it lies hidden and dormant for weeks or months). Though the type of hypogeal germination of *A. Bidwilli* is similar to that of the South American species in its hypogeal characters, it differs in the fusion of the cotyledons, the enclosure of the plumular apex, which is undeveloped, and the possession of a long period of subterranean dormancy in seedling development before the young plant appears above ground.

Cotyledon number, as at present known, ranges from 2 to 4 in both *Eutacta* and *Columbea* (Butts and Buchholz, 1940) and is therefore valueless as sectional character.

Cone-scale types

The striking and well-contrasting cone-scale types in species of *Araucaria* have been used by all authors in both early generic and later sectional divisions of the group and are excellent and reliable characters. The genus possesses three general types of bract-scale units: the nut type, the samara type, and the free-seed type. In the first two the seed is permanently retained as a part of an indehiscent structure; in the third, a hard-shelled 'seed' is set free from the bract-scale unit at maturity.

The nut type is characteristic of the South American species of *Columbea*, *A. araucana*, and *A. angustifolia*. This fruiting structure is subcylindric or narrowly obovoid, hard-shelled, and swollen by the enlarged seed within (Wilde and Eames, 1948, Pl. VI, Figs. 13, 14). In the seed-bearing region it shows little external evidence of its bract-plus-fertile-scale nature. Only a slight ridge on each side marks the wings which are a prominent feature in all other species. The fertile-scale tip is free in the Chilean species but is hardly apparent in the Brazilian species.

The samara type (Pl. II, Figs. 1–11) is characteristic of the Australian, New Caledonian, and East Indian species with the exception of *A. Bidwilli*. The cone units of this samara type have broad, thin margins definitely wing-like in some species and the units fall from the trees like samaras. The wings in some species are extremely delicate. The ripened seed remains permanently 'enclosed' within the central woody part. In *A. excelsa*, *A. Cunninghamii*, and *A. columnaris* at least, although the seed has a thick, enveloping stony layer, it is never freed from the unit.

The three New Guinea species have strongly winged cone-scales (Pl. II, Figs. 7, 10, 11); in *A. Klinkii* the wings are thin and papery (Pl. III, Fig.

13). The units of these New Guinea species, as can be seen by a comparison of the cone-scales shown in Pl. III, are broader and of a different shape than those of the Australian and New Caledonian species. In the mature bract-scale units of *A. Klinkii* the ovuliferous scale is inconspicuous and fused with the bract throughout nearly its entire length (Pl. III, Fig. 13); only a small tip, 2–3 mm. long, is free. The seed is retained on the scale when it is shed from the cones.

A. Bidwilli, so far as known, stands alone in its dehiscent, free-seed character. The bract, fertile scale, and seed in this species are fused, as in the samara-bearing species, into a broad, winged structure, but the wings are thick and woody. The fertile scale-tip is prominent. The structure as a whole is heavily woody throughout, the wings being unlike those of the samara type in this respect; the unit, with this make-up, can hardly 'function' as a samara. From this heavy fruiting scale a 'seed' is set free at maturity. The 'seed', as described in the authors' preceding paper (Wilde and Eames, 1948), is not a true seed but a seed plus, on its dorsal side, accessory fertile-scale tissues, and minus, on its ventral side, the outer layer of its integument which becomes papery and is easily ruptured at maturity.

The freeing of a seed-like structure in *A. Bidwilli* is a taxonomically important character in a genus in which indehiscent fruiting units are characteristic. This is accompanied by the morphologically most important character of a double vascular supply for the bract-scale unit: one trace to the bract, giving a series of normally oriented vascular bundles in the dorsal part of the unit, and a pair of traces, branch-like in origin, giving rise to a series of inverted bundles above the dorsal series (Worsdell, 1899; Eames, 1913; Wilde and Eames, 1948). All other species so far studied have only a common trace to the bract-scale unit. This single trace represents the fused traces of the two organs.

Three new species of *Araucaria* have recently been described from New Caledonia (Buchholz, 1949): *A. humboldtensis*, *A. biramulata*, and *A. Bernieri*. These are all apparently members of Section *Eutacta* and are not further discussed here, since Buchholz will doubtless soon describe and discuss them in detail.

Evaluation of sectional treatment

It was at first believed possible by the authors to return to Antoine's division of the genus into sub-genera, but on a different basis, that of two distinct vegetative types, present in *Araucaria* as in many other coniferous genera. The leaf-reduction type with terminal male cones might characterize one subgenus in contrast with the branch-reduction type with axillary cones the other. Under these two subgenera the species might then be grouped into sections on the basis of cone-scale types. Certain difficulties arise, however, in the distribution of other characters among the species. As more species are discovered, moreover, these difficulties may become multiplied. Such a classification would not allow flexibility and might not represent natural affinities.

The New Guinea species, for example, are probably more closely allied with species of *Eutacta*, which they resemble in their winged cone-scales, epigeal germination, and purely bracteate male cones, although in some vegetative characters and cone position they resemble the large-leaved, branch-reduction type characteristic of *Columbea*. Actually their branch-reduction is not as advanced as that in the *Columbea* species. *A. Muelleri* and *A. Rulei* also offer difficulties. Although they approach the *Columbea* species in leaf size, cone size, and number of microsporophylls, their winged cone-scales and terminal cones show their *Eutacta* affinities. Certainly these species need further investigation and, when they are better known, it may be found that at least *A. Muelleri* does not belong in either *Columbea* or *Eutacta*. There is no agreement at the present time as to where these two species should be placed. Finally, *A. Bidwilli*, which also possesses some characters of each group, stands alone in its double vascular supply for bract and scale, and in its large 'seed', which, unlike that of all other known species of *Araucaria*, is shed from the scale.

It seems preferable, therefore, to delimit more sharply the existing sections of *Eutacta* and *Columbea* by basing them on more clear-cut and reliable characters. With the sections *Eutacta* and *Columbea* more clearly defined, the New Guinea species and *A. Bidwilli* stand apart. The former have been placed in the new section *Intermedia* (White, 1947) and it is here proposed to remove *A. Bidwilli* from the *Columbea* section, and to erect a new section, designated *Bunya*, to contain *A. Bidwilli*. At present this species is the only known member of the section. The name *Bunya* is derived from the name 'bunya bunya' or 'banya banya' given to this plant by the Australian natives, with whom the seeds form an important food. *A. Bidwilli* stands apart from all other known species in that the fertile cone-scale unit is not an indehiscent, fruit-like structure but sets free at maturity a seed-like structure; and in that the vascular supplies to bract and fertile scale arise independently from the cone-axis vascular cylinder. Its cotyledons are fused proximally and their elongated bases form a hollow tube which carries the undeveloped plumule, buried in a dormant tuber, some distance from the seed-coats.

Characters and Composition of Sections

EUTACTA Endlicher

Leaves reduced, thick and often keeled, imbricate and usually erect.

Male cones terminal, solitary; female cones on long peduncles.

Ovulate cone-scales samara-like, thinly winged, indehiscent, the seed retained on the scale at shedding; vascular supply of bract-scale unit single at source.

Germination epigeal. Cotyledons subsessile, freed from seed at germination. Seedling not fleshy.

A. columnaris (Forst.) Hook.
A. excelsa (Lamb.) R. Br. ex Ait.

A. Cunninghamii Ait. ex Lamb.
A. Rulei F. Muell.
A. Muelleri (Carr.) Brongn. et Gris.
A. Balansae Brongn. et Gris.
A. montana Brongn. et Gris.
A humboldtensis Buch.
A. biramulata Buch.
A. Bernieri Buch.

INTERMEDIA White

Leaves large, generally thin, flat, spreading; sometimes slightly imbricate; juvenile leaves needle-like, flat, small.

Male cones axillary; female cones axillary.

Ovulate cone-scales samara-like, with broad thin wings, indehiscent; seed retained on the scale at shedding.

Germination epigeal. Cotyledons subsessile, freed from seed coats at germination. Seedling not fleshy.

A. Hunsteinii K. Schum.
A. Schumanniana Warb.
A. Klinkii Lauterb.

BUNYA, sect. nov.

Leaves large, flat, spreading or slightly imbricate.

Male cones axillary; female cones subsessile or on short (to 2 cm. long) peduncles.

Ovulate cone-scales large, heavy, with woody wings; dehiscent, the large 'seed' shed from the scale at maturity; vascular supply of bract-scale unit double at source.

Germination hypogeal. Cotyledons long-stalked in germination, retained within seed coats, the stalks fused into a hollow cylinder. Seedling fleshy, with a long subterranean dormant period.

A. Bidwilli Hook.

Latin diagnosis

Sectio Bunya, sect. nov.

Folia magna, plana, diversa vel leviter imbricata; strobili masculini axillares; strobili foeminei axillares, subsessiles; squamae seminiferae magnae, graves, ligneis alatis, dehiscentes; semina magna, graves; semen decisum ex squama. Fasces vasculares squamae femineae duo in origine. Germinatio hypogea.

COLUMBEA Endlicher, Emend., Wilde and Eames

Leaves large, generally thin, flat.

Male cones axillary, usually two to several on foliaceous branches.

Ovulate cone-scales nut-like, wings absent, indehiscent, the seed retained on the scale at shedding; vascular supply of bract-scale unit single at source.

Germination hypogeal. Cotyledons long-stalked in germination, retained in seed coats, stalks not fused. Seedling fleshy, without a long subterranean dormant period.

A. araucana (Molina) K. Koch.
A. angustifolia (Bertol.) O. Ktze.

Key to Sections, based on Mature Plants

A. Male cones terminal; leaves small, acicular or scale-like, often keeled, imbricate; ovulate cones terminal on stout peduncles, several to many cm. long; ovulate cone-scales light, samara-like, thinly winged; germination epigeal; cotyledons freed from seed coats at germination . . . . EUTACTA

A. Male cones axillary; leaves large, spreading or more or less imbricate, flat; ovulate cones axillary, subsessile or on short peduncles 0·5–2·0 cm. long.

  B. Ovulate cone-scales winged

    C. Cone-scales samara-like, with broad, papery wings; seed small, retained on scale; germination epigeal; cotyledons freed from seed coats at germination . . . . INTERMEDIA

    C. Cone-scales heavy, with thick, woody wings; 'seed' large, heavy, shed from scale; germination hypogeal; cotyledons with long fused bases retained in seed coats at germination . . . . BUNYA

  B. Ovulate cone-scales not winged, nut-like; seed retained on scale; germination hypogeal; cotyledons retained in seed coats, with long free stalks at germination . . . . COLUMBEA

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DESCRIPTION OF PLATES

PLATE II

Illustrating Associate Professor M. H. Wilde and Professor A. J. Eames' article on 'The Ovule and "Seed" of *Araucaria Bidwilli*. II. Taxonomy'

FIGS. 1–11. Bract-scale units of various species of *Araucaria*. Natural size.

FIG. 1. *A. columnaris*. After Brongniart et Gris (1871, pl. 14, '*A. Cookii*').
FIGS. 2, 3. *A. Muelleri*. After Brongniart et Gris (1871, pl. 16).
FIGS. 4, 5. *A. Rulei*. After Brongniart et Gris (1871, pl. 16).
FIG. 6. *A. excelsa*. Redrawn from Planchon (1877, 2304–5).
FIG. 7. *A. Klinkii*. After Lauterbach (1913, p. 49).
FIGS. 8, 9. *A. Balansae*. After Brongniart et Gris (1871, pl. 13).
FIG. 10. *A. Hunsteinii*. After Warburg (1900, Taf. X).
FIG. 11. *A. Schumanniana*. After Warburg (1900, Taf. X).

PLATE III

FIGS. 12–13. *A. Klinkii*.

FIG. 12. Specimen bearing axillary male cones near end of vegetative branch. Young leaves of growing branch tip are visible at right between the cones. (×½.)

FIG. 13. Bract-scale unit showing thin, papery wings and inconspicuous ovuliferous scale, with only 2–3 mm. at the tip free. (×1.)

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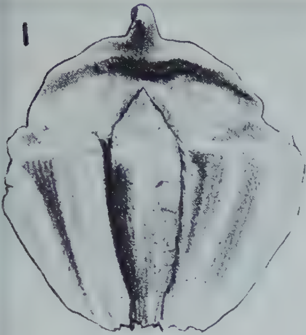

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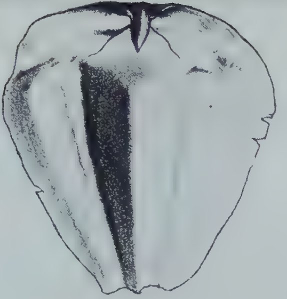

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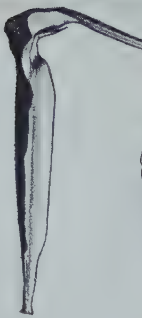

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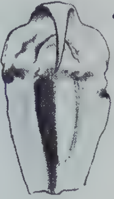

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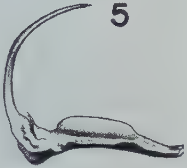

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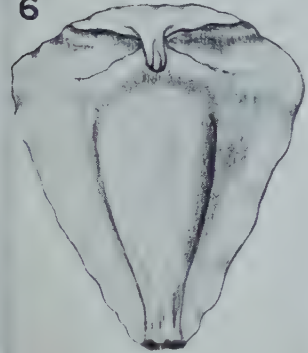

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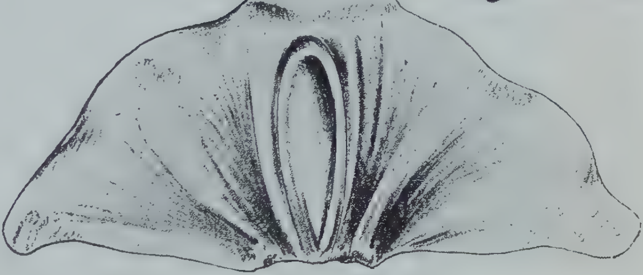

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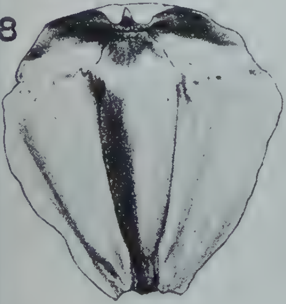

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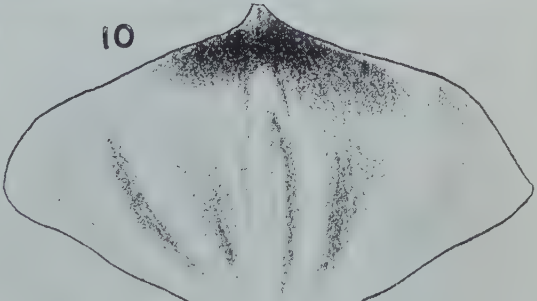

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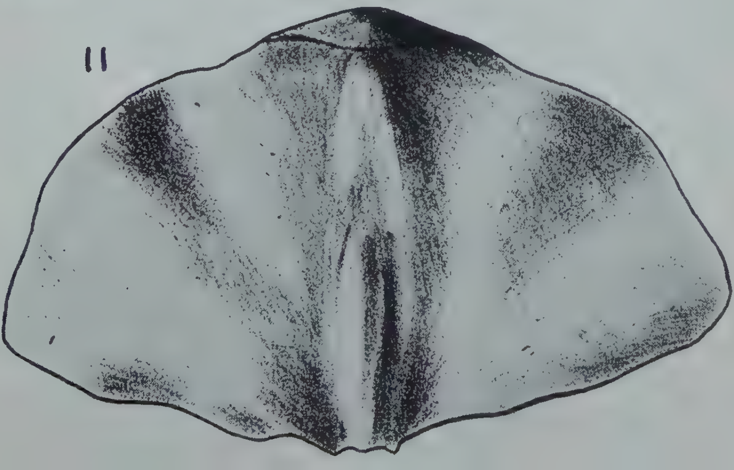

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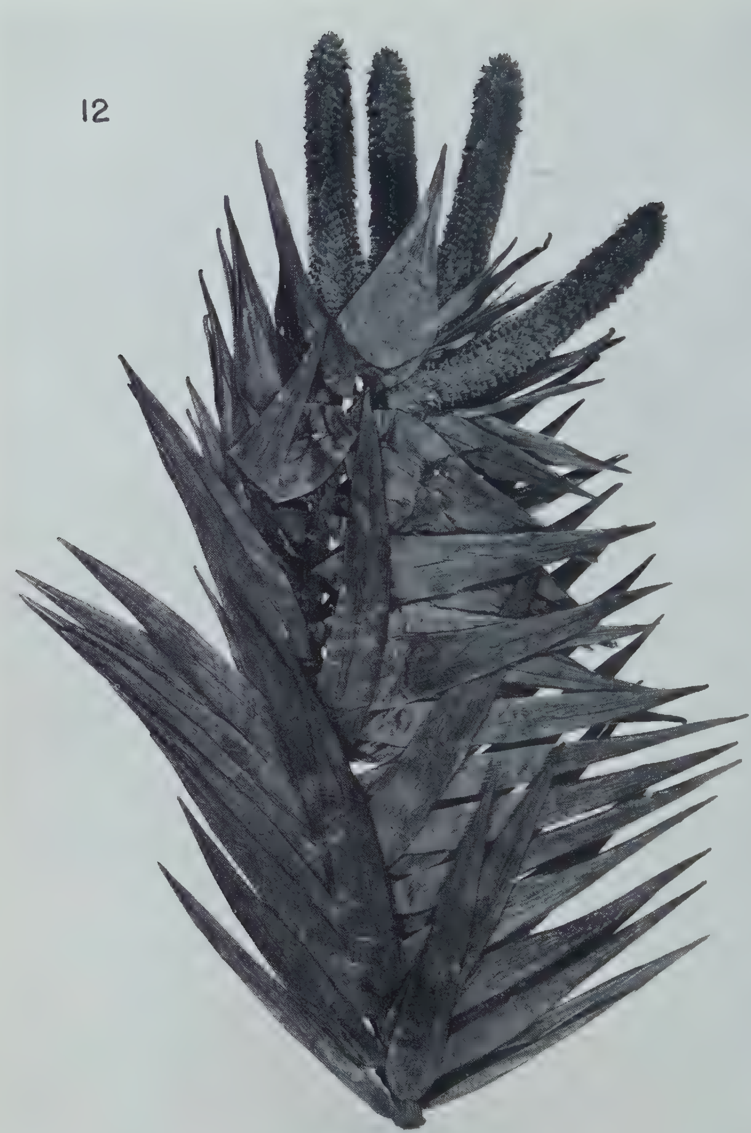

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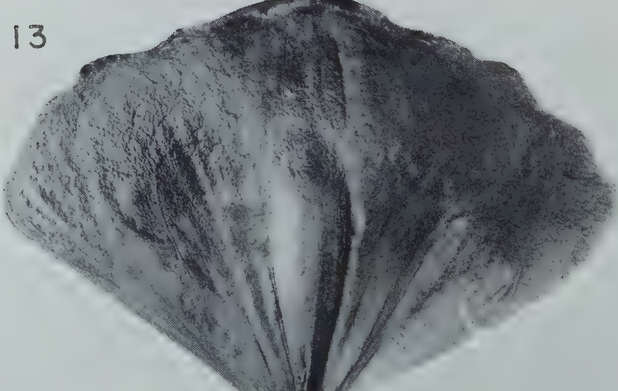

Colchicine Effect and the Production of Abnormal Spermatozoids in the Prothalli of *Dryopteris subpubescens* (Bl.) C.Chr. and *Goniopteris prolifera* Roxb.

BY

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With Plate IV and four Figures in the Text

ABSTRACT

Dilute colchicine solutions have been found to modify the early stages of germination of spores in the ferns *Dryopteris subpubescens* and *Goniopteris prolifera*. When subsequently grown on soil the prothalli continued to grow abnormally and produced abnormal sperms. Certain prothalli in both species produced large sperms, possibly being diploid, and others and less normal ones produced giant possibly tetraploid sperms.

Monstrous sperms were noted in which two, three, or four bodies were all united in front, or more rarely where the body forks into two heads.

INTRODUCTION

THE effect of colchicine on the spore germination of *Goniopteris prolifera* and *Dryopteris subpubescens* is reported. These two fern species belong to the family Aspidiaceae (Copeland, 1947). Both the species occur in a wild state along water-channels in shady environments at Amritsar, Panjab. The former is native of the plains, while the latter, which normally grows between 3,000 and 4,000 ft. in the sub-Himalayan tracts, seems to have been brought down by canal water. The ripe spores of both the species were collected on September 30, 1949.

The spores are light brown, wedge-shaped, and with three spore coats characteristic of ferns belonging to this family. In *D. subpubescens* the perisporium is better developed than in *G. prolifera*. The thickenings on the exine, which are in form of irregular meshes, are also more marked in *D. subpubescens* than in the other species. Inside are present numerous fat globules of various sizes.

The spores were germinated on half-strength of normal Knops solution (Chamberlain, 1916) in sterilized Petri dishes on October 18, 1949. This acted as control. Simultaneously other cultures were started on the same strength on Knops but containing 0·1, 0·2, 0·4, and 0·5 per cent. colchicine. All the cultures were kept in diffused light near a window. The maximum

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range of temperature during the period of experiment varied from 60° F. to 83° F.

OBSERVATIONS

In the control the spores sprouted in 3 days. The rupture occurred at the ridge joining the two flat surfaces, resulting in the emergence of a germ papilla which soon developed abundant chloroplasts. Laterally a rhizoidal cell was cut off which grew out to form a thin smooth rhizoid. This rhizoid at first contained a few small chloroplasts which disappeared after a short time.

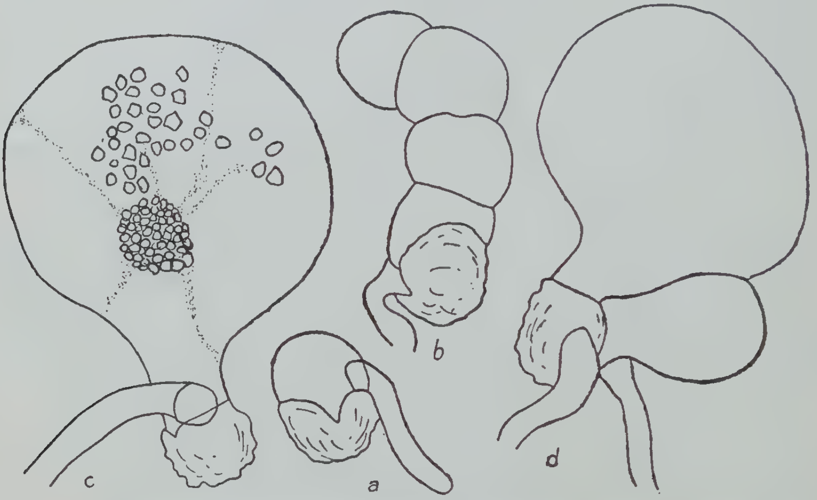

FIG. 1. *Dryopteris subpubescens*, sporelings germinating, *a* and *b* in Knops (half concentration), *c* and *d* in this solution with added colchicine. (All × 325.)

In the germ papilla numerous chloroplasts closely surrounded the nucleus, forming what is termed hereafter in the body of the paper as a nucleo-chloroplast sphere. The further growth of the sporelings resembled that of other Leptosporangiate ferns. A filamentous protonema of two to four cells developed in 6 days, and in 12 days expansion occurred in a number of prothalli by the formation of a two-sided apical cell formed in the usual fashion which segmented alternately on either side. In 3 weeks the prothalli had developed a shallow notch at the region of the two-sided apical cell in a number of prothalli. The early stages of development are seen in Text-fig. 1 *a*, *b*.

Reactions of germinating spores to colchicine cultures were more or less similar in both the species in the different concentrations of the drug. The sprouting was delayed. In 0·1 and 0·2 per cent. concentration it occurred on the 6th day (3rd day in the control), while in higher concentrations it was further delayed. In almost all cases when sprouting occurred a rhizoidal cell was cut off by a definite wall as in the control.

After 3 weeks' growth on 0·2 per cent. C-Knops the germ-cells became intensely swollen and looked like balloons (Text-fig. 1 *c*, *d*). Chloroplasts were present in abundance, scattered as well as surrounding the nucleus, forming a larger nucleo-chloroplast sphere than that observed in the cells of control cultures. The chloroplast-size was similar to that of the controls, but chloroplasts were more numerous. It was not possible to determine the constitution of the nuclei, but judging from the comparative sizes of the nucleo-chloroplast spheres one could imagine them to be polyploid in colchicine-treated cultures. Often the germ-cell became divided into two- or three-celled filaments as if unaffected by the presence of the drug and then the upper of these became gigantic. The rhizoids, which as a rule were separated from the germ-cell by a definite wall, were in all cases deformed, swollen, and irregularly branched. When there were two or more branches only one contained the nucleus, while the others were enucleate or the nucleus lodged itself at the point of branching. These sporelings presented a strong contrast to the sporelings of the same age in the controls which by now had started expansion.

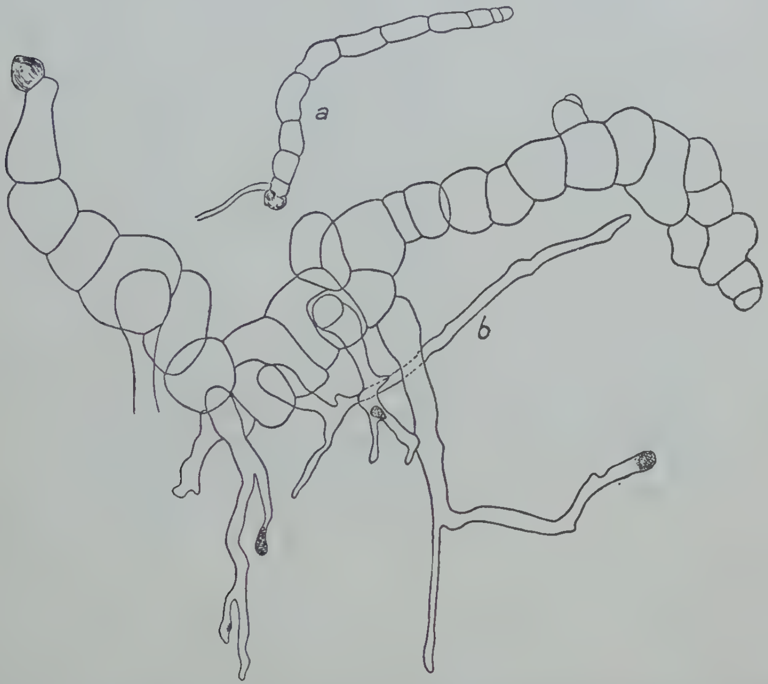

FIG. 2. *Goniopteris prolifera*: *a*, a haploid filament; *b*, a presumably polyploid filament. The dark shaded ends of the rhizoidal branches are undergoing senescence. (Both × 58.)

Another interesting point observed in colchicine cultures was the occasional presence of chlorophyllous rhizoids. The rhizoidal cell when cut off contained abundant chloroplasts, and in its further growth to form an elongated rhizoidal tube the chloroplasts multiplied. These rhizoidal tubes were as green as the germ-cells. Chlorophyllous rhizoids in some cases became swollen and produced secondary branches. Such chlorophyllous rhizoids were never

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observed in the control cultures. It would be interesting to find out if these chlorophyllous rhizoids could assume the function of the main filament if the germ-cell was destroyed.

A few sporelings were also observed in which a septum between the rhizoid and its parent cells was absent, so that the cavity of both was continuous. The chloroplasts simply moved into the rhizoidal tubes and multiplied there, forming chlorophyllous rhizoids.

The cultures of both the species after 3 weeks' growth on 0·2 per cent. C-Knops were transferred to soil in large Petri dishes, and simultaneously the sporelings from pure Knops were also transferred to soil and placed side by side under similar conditions to act as controls.

After 2½ months' growth on soil the prothalli in the controls expanded into typical heart-shaped structures. The two lobes were equal in size and of the same age. The two-sided apical cell had divided into the usual brick-shaped meristem in the apical notch. The cell walls were thin without any collenchymatous thickenings. Unicellular glands characteristic of the Aspidiaceous ferns appeared on the margin, the dorsal, and the ventral surfaces. Usually a typical cushion developed. Besides these cordate prothalli many remained ameristic and developed antheridia copiously. Antheridia as well as archegonia developed on flattened prothalli on the under surface in the region of the cushion, so that the prothalli were monoecious. The structure of the antheridium was as usual in the Leptosporangiate ferns. There was a basal cell, a ring cell, and an opercular cell. The number of spermatocytes varied from 30 to 40 per antheridium. Its dehiscence occurred either by the throwing off of the opercular cell, which degenerated after a short interval, or by its rupture at its position.

The spermatocytes escaped from the antheridium one by one, and after remaining quiescent for a varying period from a few seconds to 1 or 2 minutes, their outer walls gelatinized and a sperm wriggled out of each spermatocyte. These 'haploid' sperms had darting movements. They were fixed over osmic acid fumes, stained in 1 per cent. acid fuchsin, and mounted in balsam after passing the slides through the usual grades of alcohol and xylol. This procedure gave excellent results.

The sperms in both *D. subpubescens* and *G. prolifera* have practically a similar structure which conforms in plan to that of the Leptosporangiate ferns studied by Yuasa (1938). There are two and a half coils. The anterior end is truncated and somewhat narrower in *D. subpubescens* than in the other species. A distinct lateral bar *l* is visible at the margin of the truncated end by its affinity to take deeper stain (Text-figs. 3 *a*, 4 *a*). The 'Border Brim', however, could not be observed. The blepharoplast band which stains rather feebly with acid fuchsin, extends only half the length of the sperm and is broadest in the middle but narrower towards the two ends. The numerous cilia are borne only on its outer surface. They arise irregularly but roughly in four rows in the broadest region, decreasing to three, two, and one towards the ends. At the extreme anterior end the cilia are lacking. The swollen bases of cilia are clearly seen. The nuclear band, which stains deeper than the blepharoplast, does not extend to the apex of the sperm but stops short a little below it. It does not stain homogeneously in well-differentiated preparations but shows unstained gaps here and there, indicating meshes between the chromonema threads of which it is composed. At the tail end a vesicle representing the remnants of the cytoplasm of the spermatid is often present.

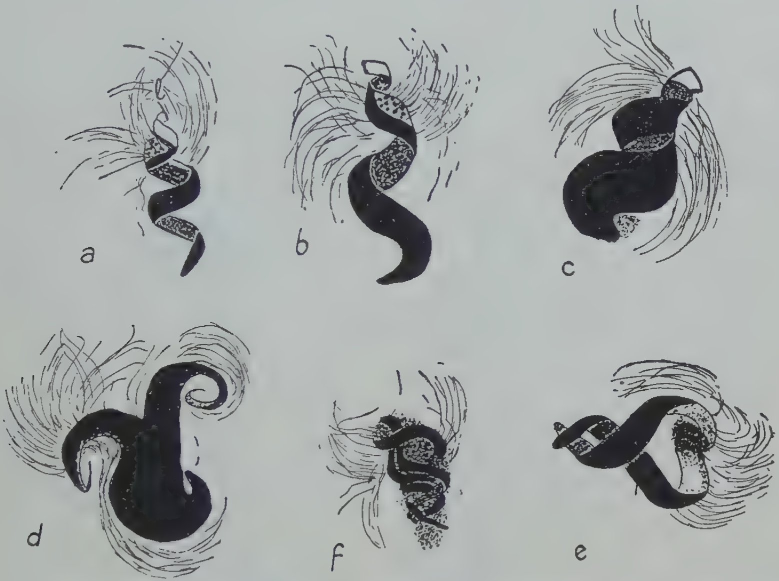

FIG. 3. *Goniopteris prolifera.* *a*, *b*, *c*, respectively, are normal, large (B), and giant (C) sperms; *d*, double-headed giant sperm; *e*, large twins; *f*, triplets. (All × 1000.)

The sperms that escaped from colchicine-treated prothalli of *G. prolifera* after 2½ months' growth on soil are of two types, both bigger in size than the haploid sperms of the normal type. Three sperm types are therefore distinguished, here called the normal type, the 'large' or B type, and the 'giant' or C type. The writer imagines that the 'large B' type may be diploid and the 'giant C' type may be tetraploid, but there is no direct evidence of their chromosome number. These abnormal sperms are shown in Text-fig. 3, together with the normal, haploid sperm. The 'giant C' type has longer cilia in a thicker bunch than the normal, the nuclear coil is thicker, and the vesicle at the posterior end is larger. The movements of the three types are different; the normal ones dart about; the 'large B' type move less quickly, and the 'giant C' type are awkward and sluggish in their movements.

In *D. subpubescens* treated with colchicine various abnormal sperms were also seen. There were large ones which seem to correspond to the 'large B' type of *G. prolifera*, but I saw none corresponding to the 'giant C' type. On the other hand, there was an interesting series of monstrous sperms which may be called twin, triplet, and quadruplet.

The twin sperms are each of the same size as a normal (haploid) sperm, so this is imagined to be their chromosome number, though again there is no direct evidence. They are usually united at the anterior end, but although the rest of the two coiled bodies are free they are sometimes intercoiled or else free from one another. Text-fig. 4, *j*, shows a triplet sperm with two bodies intercoiled, one separate. A good many probable quadruplets were seen, but here the bodies are difficult to make out fully (Pl. IV, Figs. 4, 5, 6, and Text-fig. 4 *d–h*).

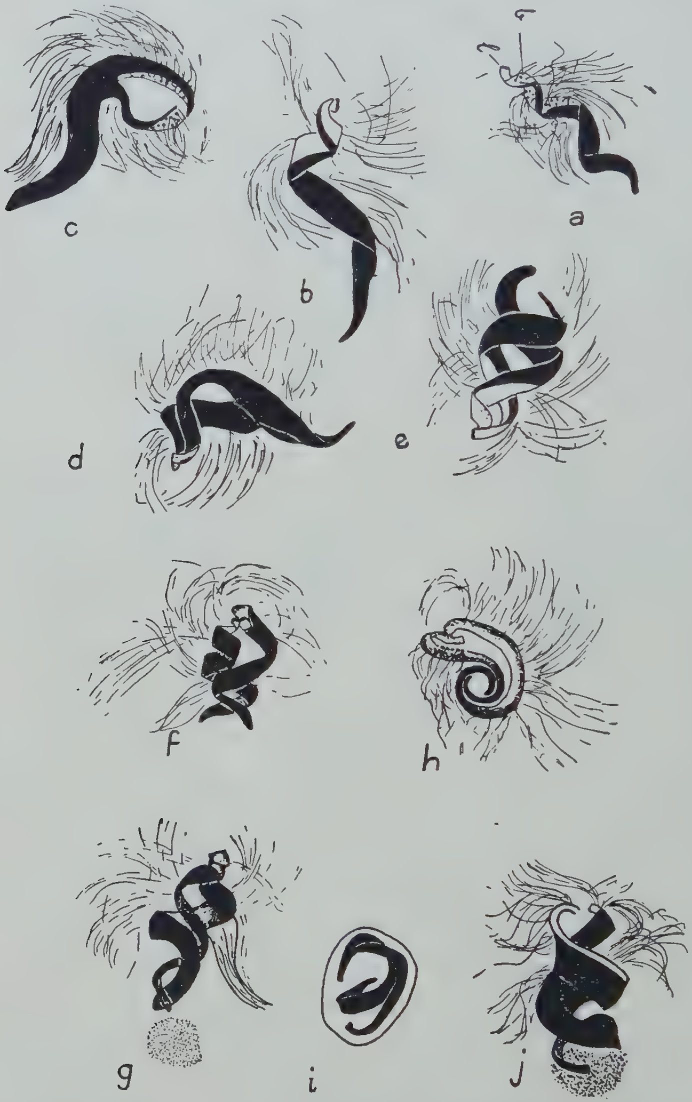

FIG. 4. *Dryopteris subpubescens*. *a* and *b*, normal and large sperms; *c*, double-headed large sperm; *d–h*, twins; *i*, twins within a spermatocyte; *j*, triplets. (All × 1000.)

Another kind of monster (observed in both species) is a two-headed sperm with a single body; and the size of these monsters may agree with either the 'large B' type or with the 'giant C' type (Pl. IV, Fig. 7, and Text-figs. 4 *c* and 3 *d*).

I feel sure that these monster sperms have each been formed from a single mother cell; indeed I have frequently observed their release from a single cell, and Text-fig. 4, *i*, shows a twin sperm developing in a single cell. The cytology of the production of these monsters is, however, unknown.

The only case known to me in the literature of a double-headed sperm, comparable to what I have described here, is in man (Kostoff, 1946), where it is ascribed to abnormal spermatogenesis.

The prothalli of *G. prolifera* which produced the 'giant C' type sperms never grew beyond a filamentous stage or at best a prothallus 3 cells wide, but they were thick, dark green, and sturdy looking (Text-fig. 2). Their rhizoids are branched and deformed just as in the earliest stages while still being treated with colchicine. Their nuclei were larger than in the normal (haploid) prothallus. The glands were gigantic compared to those on the normal prothallus and were often bifurcate or trifurcate. The antheridia likewise were gigantic and differed from normal antheridia in possessing a prominent nipple like bulging of the opercular cell. It was not found possible to count their chromosomes with accuracy, but a few metaphases were seen in the young antheridia in which it is evident that there are a good many more than the normal number of chromosomes (Pl. IV, Figs. 2, 3).

The prothalli which gave the 'large B' type sperms were of much more normal appearance. After 3 months' growth they have become the usual cordate shape, and their rhizoids are much less branched than in those giving the 'giant C' sperms. In certain respects they are intermediate between the normal and 'giant C' type—that is in size of the cell, size of the nuclei, size of the unicellular glands and of the rhizoids.

ACKNOWLEDGEMENTS

My thanks are due to Mr. R. S. Pathania for taking the microphotographs illustrating this paper, and to Prof. T. M. Harris, F.R.S., for criticizing and revising the manuscript.

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EXPLANATION OF PLATES

Illustrating P. N. Mehra's article on 'Colchicine Effect and the Production of Abnormal Spermatozoids in the Prothalli of *Dryopteris subpubescens* (Bl.) C.Chr. and *Goniopteris prolifera* Roxb.'.

Goniopteris prolifera (1–3)

FIG. 1. Normal (A), large (B), and giant (C) sperms.

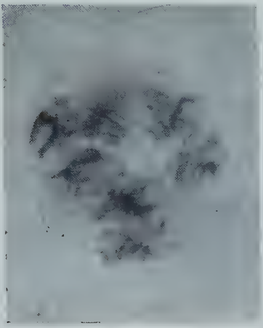
FIG. 2. Metaphases of spermatogenous cells in antheridium of a haploid gametophyte.

FIG. 3. Anaphase of primary spermatogenous cell of antheridium of a probably tetraploid filament.

Dryopteris subpubescens (4–7)

FIGS. 4, 5, 6. Twins.

FIG. 7. A double-headed diploid sperm.



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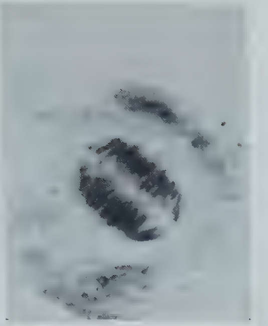

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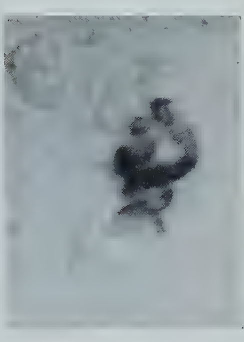

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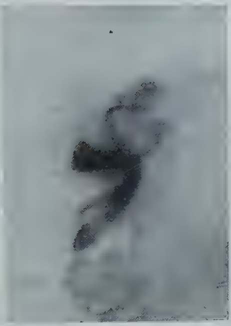

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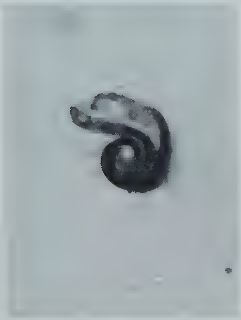

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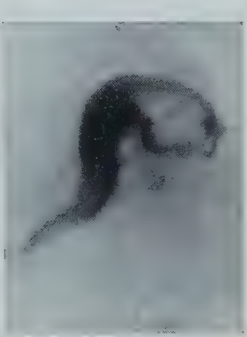

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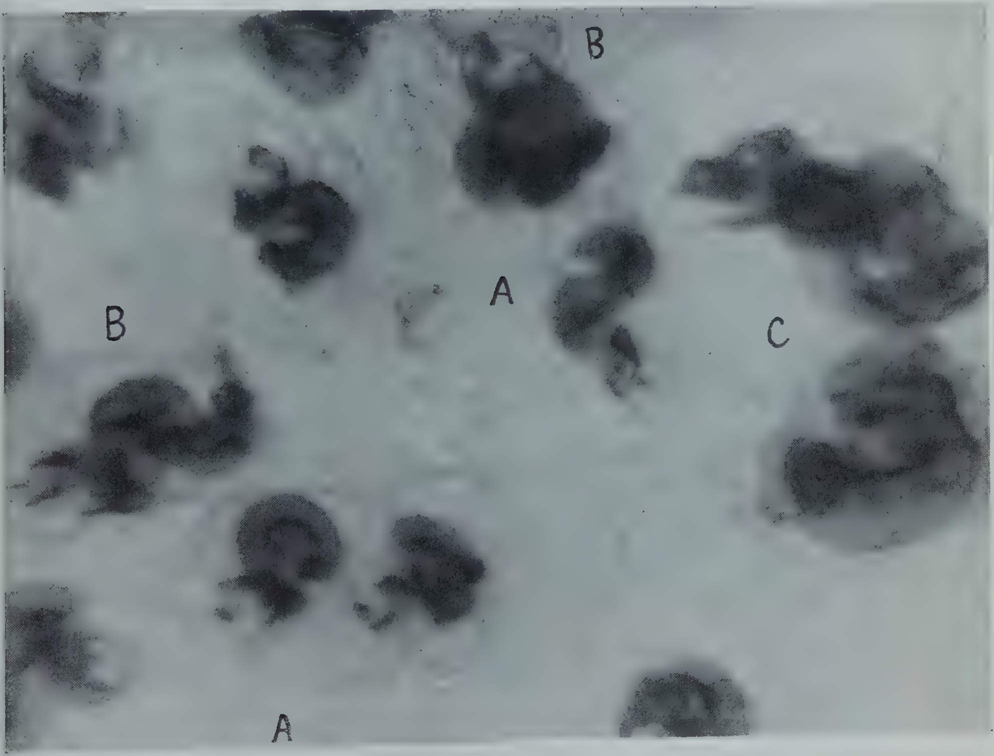

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P. N. MEHRA

Investigations on Growth and Metabolism of Plant Cells

I. New Techniques for the Investigation of Metabolism, Nutrition and Growth in Undifferentiated Cells

BY

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AND

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With Plates V and VI and nine Figures in the Text

ABSTRACT

This paper describes a technique for comparisons of the metabolism of rapidly growing and non-growing tissues. The requisite conditions for such studies are discussed. Active tissue growth is obtained by using aseptic conditions and a complete nutrient medium with coco-nut milk added. Rapid diffusion and gas exchange is obtained by using special tubes in which the tissue and medium are rotated in the presence of a large volume of air. Modifications are described which enable various types of metabolic treatment to be used, including the use of tracer carbon. The tissue principally described is carrot phloem, but various other possibilities including the use of micro-organisms are envisaged.

AN ultimate aim of the plant physiologist is to understand growth. This entails not merely the recognition and description of the several vital functions and processes, but an adequate comprehension of their integration into a harmonious working machine.

The methods of cell physiology have often tended to isolate a given process or vital function for separate investigation, and to treat it as remotely as possible from what are often called the 'complications due to growth and metabolism'. This phase is passing. Modern trends in the investigation of respiration, protein and carbohydrate metabolism, salt and water absorption, permeability and secretion, recognize the interrelations of all these processes. This was the dominant point of view in an earlier series of papers (for references see Steward, et al., 1943).

In this field of cell physiology the following questions may be posed: What constitutes the essential physiological attributes of a cell that can grow and divide? In what physiological terms may the differences between living, but non-dividing, parenchyma cells and those which actively proliferate be expressed? Why does metabolic energy in some cells apparently run to waste,

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whereas in others it is canalized towards the production of new substance and brings about processes which increase the free energy of the system? The challenging importance of such problems and concepts has stimulated much of the work in this group of papers. Much of the experimentation was based upon certain new devices by which growth, nutrition, and metabolism in plant cells may be investigated, and the design of these is here described.

In the attempt to deal at the cellular level with selected problems, the physiologist has made full use of such autotrophic unicellular green algae as *Chlorella* and *Scenedesmus* and such heterotrophic organisms as yeasts. Bacteria, especially the larger sulphur bacteria and mammalian blood cells, have all been prominently employed. The resort to large coenocytic structures, such as the internodal cells of the Characeae or the large vesicles of the Valoniaceae, is a familiar device where problems demanding analysis of internal aqueous fluids arise. Conspicuously vacuolated cells wherever they occur in the plant kingdom, i.e. from filamentous algae to the epidermis of flowering plants, have also found their use wherever the techniques based on the osmotic behaviour of cells apply. In the investigation of intermediary metabolism, the use of tissue slices of surviving animal organs has become a staple technique and this has found its counterpart in the use of tissue slices of plant storage organs as a material on which to investigate the respiration and metabolism of these cells and particularly the interaction of these physiological processes with salt and water absorption. (A series of papers, for references see Steward and Preston, 1941, dealt with the biochemistry of salt absorption.)

In seeking, therefore, for new methods and new materials upon which to attack the ultimate problems of the metabolism of growing and non-growing cells, and on which to investigate the connexions between such diverse and fundamental processes as respiration, protein synthesis, and salt accumulation, it was found that most of the tried and tested techniques and materials left much to be desired.

A system suitable for these general purposes should have several characteristics as follows:

1. It should be composed of cells which can be put reversibly and at will in the condition in which they will, or will not, multiply by cell division in order that the metabolic characteristics of the growing and non-growing cells may be contrasted.
2. It should be derived from sources which are easy to procure at all seasons of the year.
3. It should permit the investigations to be carried out under aseptic conditions and thus allow the use of organic nutrient media for long periods of time.
4. It should submit the cells or tissue to experimental conditions which effectively control the variables that determine their behaviour so that accurate quantitative data may be obtained.

5. It should preferably consist of cells which are as representative as possible so that the results obtained may not be unduly restricted in their application.
6. It should be readily adaptable to techniques which involve radio-active isotopes since these may be required at some stages of the investigations.

These objectives were fulfilled by extending the known techniques of plant tissue culture in ways which will now be described.

The cultivation under aseptic conditions of explants from selected tissues or organs of higher plants has been energetically pursued by numerous investigators (Robbins, 1922; White, 1934, 1939; Gautheret, 1939–41; Nobécourt, 1939; Hildebrandt, 1945–6) chiefly, however, in the attempt to demonstrate the inherent ability of the cells to grow and the nutritional requirements which are compatible with 'potentially unlimited growth'.

However, the possibilities in the tissue-culture technique for the investigation of problems of growth and metabolism have not been fully developed. As commonly employed the tissue-culture method results in irregular growths, highly variable even when small and apparently uniform pieces are subcultured from the same mass (Caplin, 1947). Such cultures, commonly grown on agar and propagated by repeated sub-cultures, are rarely suitable for precise quantitative physiological experiments. The technique developed, therefore, aimed at the use of material which would be reproducible and uniform in its behaviour and which could be grown in aqueous solution. It is true that the apparatus adopted was devised specifically for the growth of a particular plant tissue culture, but in use it was found to be more general in its application and, as it is now used, it constitutes a general method of approach to a variety of problems in which growth and metabolism are concerned.

In the selection of a suitable tissue culture for the general purposes which were in mind, there were limitations set by the then (1947) existing knowledge of the plant tissue-culture technique. At that time tissue cultures were of the following kinds:

(i) Tissue originally incited to grow by pathogens (White and Braun, 1942) although the cells of the growing culture were eventually freed from the inciting agent. In this class fall the following tissues which are commonly cultivated in laboratories where tissue culture is practised: sunflower (*Helianthus annuus*) stem, Paris daisy (*Chrysanthemum frutescens*) stem, marigold (*Tagetes erecta*) stem (Hildebrandt and Riker, 1946), &c.

(ii) Tissue in which the ability to grow and proliferate is spontaneous, such as the tobacco hybrid (*Nicotiana glauca* ♀×*N. langsdorfii* ♂) (White, 1939), and is the consequence of a particular combination of genetic factors.

(iii) Tissue in which the ability to grow and proliferate derives from cambial tissue isolated with the explants and maintained in a continuously dividing condition by some stimulant (e.g. indoleacetic acid or naphthaleneacetic acid) producing a culture which may become heterotrophic for this agent. Examples are the cambial tissue from carrot root (*Daucus carota*), Jerusalem artichoke tuber (*Helianthus tuberosum*), endive (*Cichorium endivia*), salsify (*Scorzonera* sp.) as used by Gautheret (1941), and grape (*Vitis* sp.) by Morel (1944).

(iv) The organ cultures, properly so called, are represented by the cultivation of excised roots after the procedures of Robbins (1922) and White (1934).

Though each of these systems had its merit for some purposes, none of them fulfilled all the prerequisites. Various tissues and treatments have therefore been examined, and among these the outstanding response has been that which occurred between the mature cells of the secondary phloem of carrot root cut far enough from the cambium so that they had ceased to divide, and coco-nut milk (the liquid endosperm from mature nuts). It was this combination which suggested the possibility of bringing a living tissue, normally able only to survive, or with at most a limited ability to grow, into a condition of rapid growth and proliferation. Our standard system for the study of growth was therefore developed upon the carrot culture method which is described below. It should be emphasized, however, that other organs of other plants, notably Jerusalem artichoke tuber, could probably have served equally well. These two tissues far excelled the others which were then tried in their potentiality for rapid and essentially undifferentiated growth under the stimulus of a growth factor (C.M.F.) contained in the coco-nut milk.

The effect of coco-nut milk as an active agent capable of stimulating explants from the secondary phloem of carrot root into rapid growth has already been noted (Caplin and Steward, 1949). In this work standard cylinders (diam. 2 mm., length 1 mm.), with their long axes oriented along the radial direction in the carrot, were removed from the root in such a way that the explants used were all derived from the same distance from the cambium. The basal nutrient medium was that used by White (1943) and contained salts, sugars, and a variety of accessory growth factors (glycine, thiamin, pyridoxine, and niacin); to this medium was added 0·8 per cent. agar. Cultures were grown in test-tubes. This paper showed that whereas the addition of indoleacetic acid in a wide range of concentrations over the same period (21 days) only caused the carrot explants to grow to a limited extent, the addition of coco-nut milk caused a very striking growth response, with an obvious maximum when about 15 per cent. by volume of whole coco-nut milk was added to the medium. Other work has shown that:

(1) Coco-nut milk, even after it has been heat sterilized and filtered, gives this effect.

(2) The same effect cannot be obtained by the use of such potent and general sources of vitamins and growth substances as extracts of auto-

lysed yeast, liver, tomato fruit, &c., or even extracts of carrot tissue or potato tuber tissue, nor can it be obtained by the use of the ash obtained from the coco-nut milk.

(3) The effect is not one which it has been possible to duplicate by any known combination of organic nutrients or by the addition of any known specific substances.

(4) One of us (F. C. S.), together with E. M. Shantz,[1] is isolating the active principle, and from this work it can be stated that the stimulus to carrot phloem is a thermostable, water-soluble organic constituent of the coco-nut milk which represents only a small fraction of the total dry matter.

In the work reported here, however, the nutrient medium in which the carrot explants grow satisfactorily is the basal medium of White supplemented by heat-sterilized whole coco-nut milk (usually 10–15 per cent. by volume, but depending somewhat on the activity of the batch in question). Actually considerable growth would occur even if the basal medium is omitted entirely, since whole coco-nut milk itself contains most of the staple nutrients.

The presence of a substance or substances in coco-nut milk that produce much more rapid growth, though without differentiation, of explanted tissues has now been confirmed in other laboratories (Duhamet, 1950; Duhamet and Gautheret, 1950); although Gautheret (1950) reiterates that carrot tissue 'habituated' by indoleacetic acid can be grown *slowly* in media which lack all known growth substances.

THE TISSUE-CULTURE APPARATUS

Growth of parenchyma tissue in water presents several problems. The need to aerate isolated discs of storage tissue in order to maintain their full activity in respiration, protein synthesis, and salt intake was familiar from previous work (Steward, 1932, and Steward, Berry, and Broyer, 1936). The importance of aeration in maintaining the full activity of excised roots was also familiar (Hoagland and Broyer, 1936). It is true that organ cultures of excised roots are commonly grown immersed in flasks in which the needs for oxygen are met by solution and diffusion of oxygen through a broad surface of shallow liquid. However, it was easy to show that carrot root explants *immersed* in a nutrient which contained all the essentials for growth did not grow as readily as when the explants were in air on the surface of a semi-solid medium. The obvious suggestion was that when the tissue was completely immersed it lacked oxygen, and this limitation was eventually overcome as follows.

In the first attempts to solve these problems rapid streams of air were used, a cumbersome method of aerating a large number of liquid aseptic cultures. A subsequent method was to use the tissue in a sealed tube, containing only a small volume of nutrient and a relatively large volume of air. Gas exchange was achieved by tumbling the sealed tubes 'end over end' around a horizontal axis. It was then observed that the tissue explant adhered to the glass at one end or the other of the culture vessel and so passed part of the time of each revolution exposed to air and part freely immersed in the liquid. The greater amount of growth obtained under these conditions was promising enough to lead to the design of the present culture tube which makes use of a side neck, as illustrated in Pl. V. This rotating tube, already briefly described (Caplin and Steward, 1949), has become the basis of the present technique. Its special features may be summarized as follows:

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[1] Work being carried out in the Botany Dept., Cornell University, under grant from the U.S. National Cancer Institute.

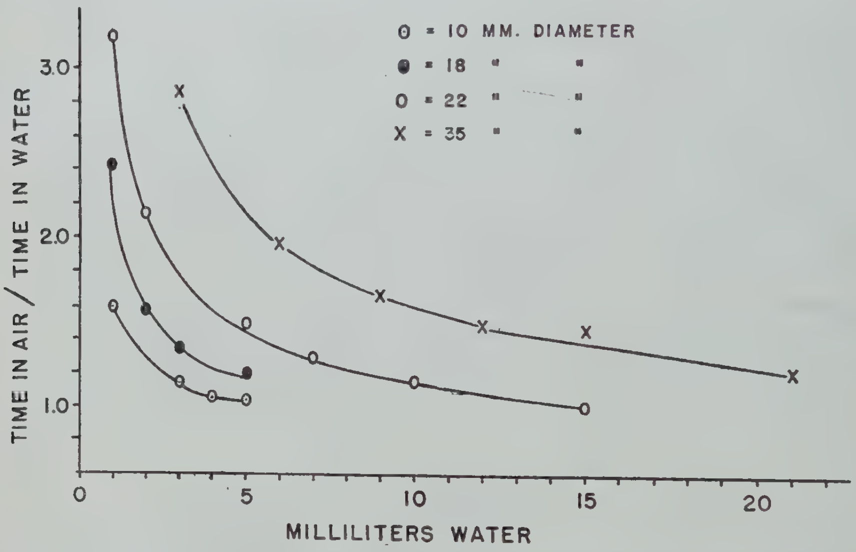


FIG. 1. Ratio of time in air to time in water of cultures in rotating tubes of different diameter containing varying amounts of liquid.

(i) When slowly revolved around a horizontal axis, the tissue is alternately exposed to air and immersed in liquid. (Actual trials have shown that between 1 and 4 revolutions per minute the speed does not significantly affect the growth rate of carrot cultures. At speeds less than this, however, e.g. ½ r.p.m., growth is significantly reduced.)

(ii) The fraction of the time of one revolution (1 min.) during which the tissue is exposed to air and to liquid is a function of the diameter of the tube and the volume of liquid it contains: this is clear from Text-fig. 1. (These curves only apply if the length of the closed tube is such that the total volume is greater than twice the volume of the liquid.) The curves show that the observed mean time in air relative to the time in water increases with the diameter of the tube and decreases with the volume of the solution it contains. A volume of solution equal to 10 ml. furnishes enough nutrients for at least 14–21 days of growth of carrot explants which weigh initially 3 mg., and in a tube of 35 mm. diameter the fractional time in air (approx. ⅔) is very effective in promoting growth. For other material than carrot tissue cultures it may be that the dimensions of the tube should be redetermined for the particular purpose in view, although the dimensions of the tubes finally adopted, which are 12·5 cm. long and 3·5 cm. in diameter, should be convenient for most purposes. They have been used by us for culturing a number of different tissues and have been employed by Dr. L. Knudson in the Botany Dept. of Cornell University for culturing latex-bearing tissues.

(iii) As the tube revolves the liquid medium flows along the tube from end to end. In so doing gaseous equilibrium between the liquid and the gas phase is maintained. The side neck (diameter 17 mm.), plugged with cotton (Pl. V), permits the atmosphere within the tube to be in equilibrium with that outside. The gas exchange through the plug is no doubt aided by the slight turbulence caused by the slow flow of liquid as the tube rotates.

(iv) The culture tubes are mounted on the periphery of a disc fixed to the rotating shaft. The tubes are attached in such a manner that they each lie along a radius of the disk and are held in position by spring clips (Pl. VI). A disk with a diameter of 18 in. can accommodate 24 tubes.

(v) The shaft which carries the disks is mounted at a slight angle to the horizontal (slope approx. 1 in 12): in this manner the side necks are slightly inclined, enough to prevent any danger of the plugs being wetted or of the tissue culture becoming lodged in the neck. In this way the maximum volume of liquid in the rotating tube may be increased if so desired.

The entire machine, called the Auxophyton,[1] consists of the following essentials:

(*a*) Two shafts which can be slowly revolved at a controlled rate (1 r.p.m.) by a suitably geared motor and driving mechanism.

(*b*) Circular discs each carrying 24 rotating or 'tumbling' culture tubes.

(*c*) Banks of fluorescent lights, so mounted that they illuminate equally the tubes carried on each of the shafts.

(*d*) The whole apparatus is contained in a room with controlled temperature (26±0·5° C.) and preferably controlled humidity.

The detailed design to achieve these ends may well vary with local conditions. Arrangements which have been found particularly satisfactory are described below and are illustrated in Pls. V and VI. The total capacity of this apparatus is 1,152 tubes: it needs, therefore, to be robust in construction.

[1] The authors have been repeatedly pressed to assign a name to this particular apparatus and technique. When first mentioned the apparatus was referred to as a klinostat because, like the traditional apparatus of that name, it did neutralize gravitational stimuli. However, the concepts that need to be embodied in a name are the following: (1) The machine or technique provides conditions conducive to rapid increase or growth of plant material. (2) It is so designed that it works smoothly and automatically. With the help of Prof. Suhr, of the Classics Department of the University of Rochester, these concepts seem to be best expressed by combining the following syllables obviously derived from the Greek: *aux(an)o-phy-ton.*

To economize space it may be mounted rigidly against a wall, although the design has been modified so that it can stand entirely upon a floor mounting with access to each side. The essential details are as follows:

The supporting frame. This is made from 2-in. angle iron, its overall length being 12 ft., height 7 ft., and depth 15 in. The top and bottom frames are welded and held together by 8 uprights, attached by bolts, and the whole is clamped rigidly to a wall. The uprights divide the apparatus into 3 bays. A guard rail (Pl. VI) protects the moving parts from external contacts.

The shafts. These are two $\frac{3}{4}$-in. cold-rolled steel parallel shafts mounted approximately 32 in. apart and sloping 1 in 12 to the horizontal. Each shaft is mounted on 4 self-aligning bearings—mounted on the uprights of the supporting frame. This divides each long shaft into 3 segments, one to each bay, and stout couplings permit each of the segments to be separately removed.

The power is supplied by a $\frac{1}{4}$-h.p. motor, with a belt drive and a suitable reducing gear to give a speed of 1 r.p.m.

Illumination. Even though the growing material is adequately furnished with sugar, light is necessary, for it affects the morphology of the cultures. In the light, tissue cultures turn slightly green, but the conspicuous feature is that they remain very compact, whereas cultures grown in the dark in time become friable. (When applied to the growth of algae, however, light is an essential part of the technique.) Illumination is furnished in each bay by banks of 40-watt fluorescent lamps, each 4 ft. long, which are mounted equidistant from the two shafts and parallel to them. The lamps are carried on supports rigidly attached to the frame, which allow them to be moved at will. For purposes of growing tissue cultures, 4 such lamps to each bank suffice (1 'white' and 3 'daylight'). In mounting the lamps and their ballasts, care was taken to ensure an unrestricted path for light (Pl. VI). This exposes the cultures to a maximum intensity of 500 foot-candles. If needed, however (e.g. for the culture of green algae, such as *Chlorella*, without sugar), the space available can easily accommodate 10 lamps in each bank (increasing the light intensity approximately $2\frac{1}{2}$ times), and additional banks may be furnished if these are desired for special purposes.

Growth of carrot explants. Growth curves of small explants of carrot root secondary phloem in basal nutrient solution (White, 1943) supplemented by coco-nut milk have been previously described for these conditions (Caplin and Steward, 1949). The original explants cut 1 mm. from the cambium weighed 3 mg.: they were placed in 10 ml. of nutrient solution and they grew rapidly after a lag period. Text-fig. 2 shows a plot, on semi-logarithmic paper, of the fresh weight against time. It is clear that the weight of the tissue increased at a slow and uniform rate for 4 days, but thereafter more rapid growth ensued and this continued exponentially until 14 days or more. Thereafter, when the cultures reached on the average about 150 mg., due to some other limitation, the exponential growth rate tended to decline. In part this may have been the natural consequence of the increased size and relatively decreased surface of the cultures.

Many trials have shown that the growth of carrot phloem cultures is far better under the conditions here described than in tubes on agar which contains the same nutrients as the liquid medium (Text-fig. 3). This can also be seen in another experiment (Table I), which indicated that the tissue grew better in the liquid cultures than on washed agar whether in stationary or in revolving tubes. There can be no doubt that carrot explants all removed from the same root at the same distance from the cambium grow better, i.e. both faster and more uniformly, under the conditions described than under the best conditions obtainable on agar medium in test tubes. The tissue grows in the dark almost as well as in the light (Table I).

TABLE I

Comparison of the Growth during 20 Days of Carrot Explants (3 mg.) on Agar and in Liquid containing Base Medium plus Coco-nut Milk

Conditions.			Medium.	Fresh weight in mg.
Light	Cultures	stationary	2% washed agar and nutrients	38·1 ± 10·2
,,	,,	,,	0·8% washed agar and nutrients	34·7 ± 7·9
,,	,,	rotated	2% agar and nutrients	41·5 ± 11·3
,,	,,	,,	Liquid medium and nutrients	98·6 ± 15·2
Dark	,,	,,	Liquid medium and nutrients	78·5 ± 13·3

Under the conditions described the coefficient of variability of the growth of carrot phloem cultures all cut from the same root is generally between 5 and 10 per cent. With occasional carrots the coefficient may be somewhat greater or less than this. The reasons for these differences in the variability in growth rate of explants from different carrots are unknown.

SUPPLEMENTARY TECHNIQUES

To make the technique applicable to the investigation of different physiological concomitants of the growth process, certain accessories have been devised, as follows:

Gas exchange. Growth in the dark (i.e. when the culture tubes are covered with black paper) is, of course, accompanied by carbon dioxide production. The total carbon dioxide produced may be collected and eventually measured by the following device. (It should be noted that this exposes the tissue continuously to a carbon dioxide-free atmosphere; trials have shown that this does not appear to limit the growth of tissue cultures.)

Attached to the side neck of the culture tubes is a standard taper (16 mm./19 mm.) ground-glass joint into which a cotton plug is inserted. The small ellipsoidal side chamber shown in Pl. V contains alkali and is attached at the standard taper joint. After any desired period, during which the carbon

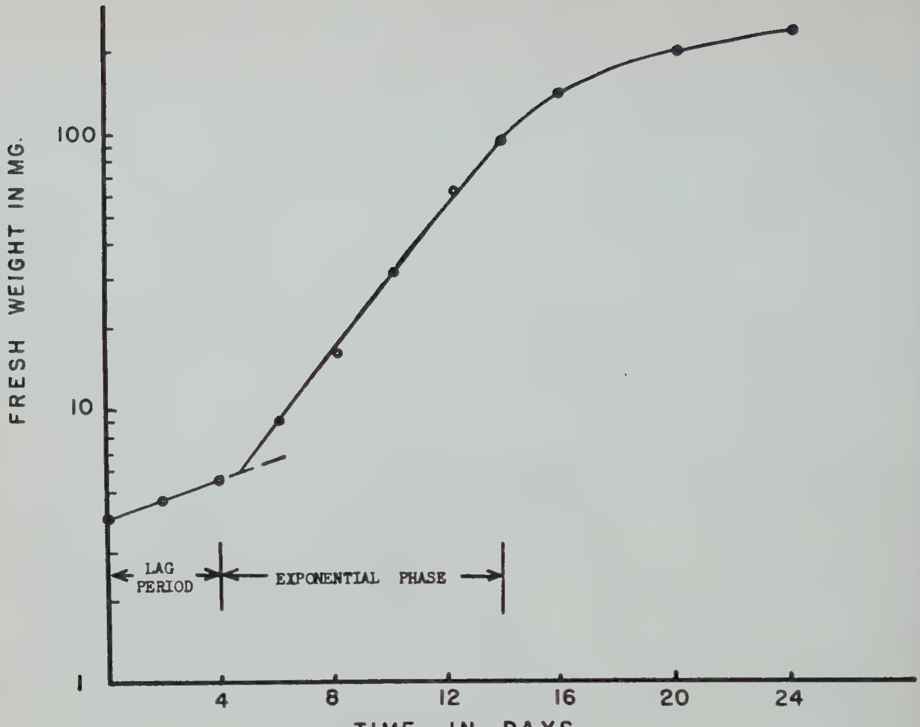


FIG. 2. Effect of time on growth of carrot explants.

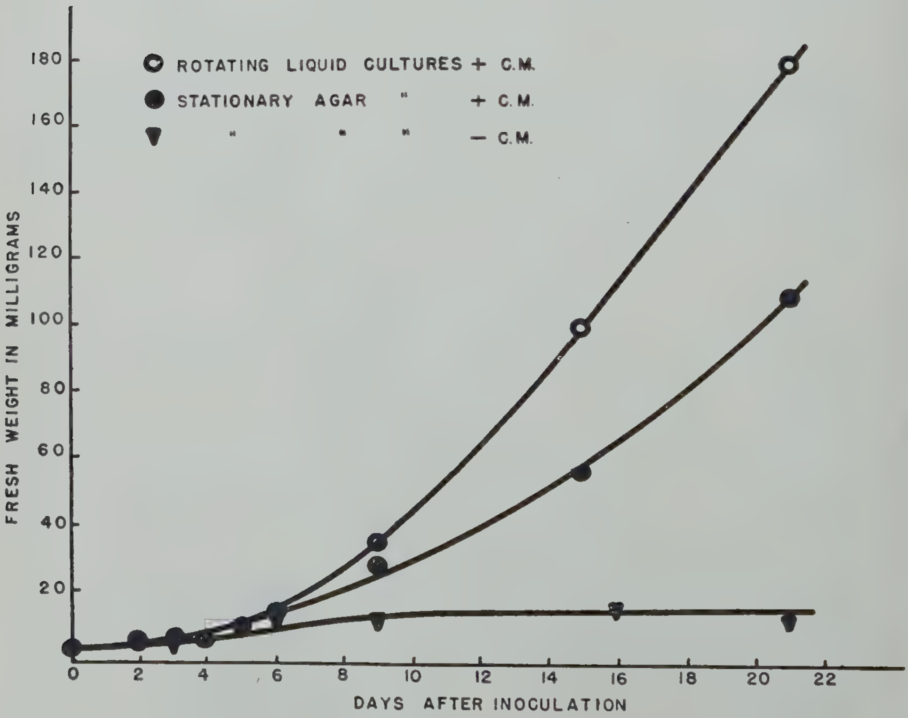


FIG. 3. Comparison of the growth of carrot explants (3 mg.) on stationary agar and in rotating liquid cultures.

dioxide produced during growth has been collected, the flask is removed, closed by a stopper which also acts as a stand (Pl. V), and the alkali is then ready for carbon-dioxide determination by any of several standard methods, depending upon the time period and quantity of carbon dioxide which has been absorbed. Double titration, change in electrical conductivity of the alkali solution measured at constant temperature, or the liberation of carbon dioxide by addition of a non-volatile acid (e.g. citric, tartaric, or succinic) in a Warburg apparatus are all possible methods, depending upon the circumstances.

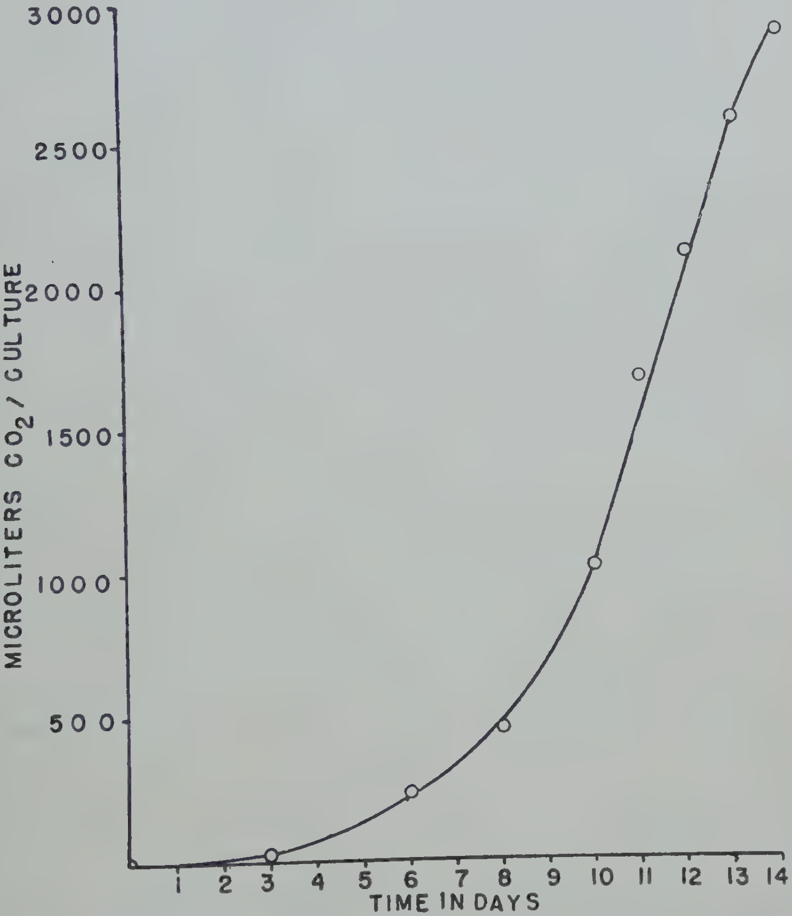


Fig. 4. The effect of time upon the output of carbon dioxide from carrot tissue cultures in nutrient medium with coco-nut milk.

Text-fig. 4 shows the time course in the cumulative carbon-dioxide production of a carrot explant exposed to nutrient medium plus coco-nut milk under the standard conditions here described. Clearly the time course of carbon-dioxide output and of growth (increase of fresh weight) are similar.

Composition of the atmosphere in contact with the cultures. The need for control of the atmosphere arises in two important ways:

(i) Oxygen tension is an important variable which needs to be investigated in relation to growth, salt absorption, respiration, &c.

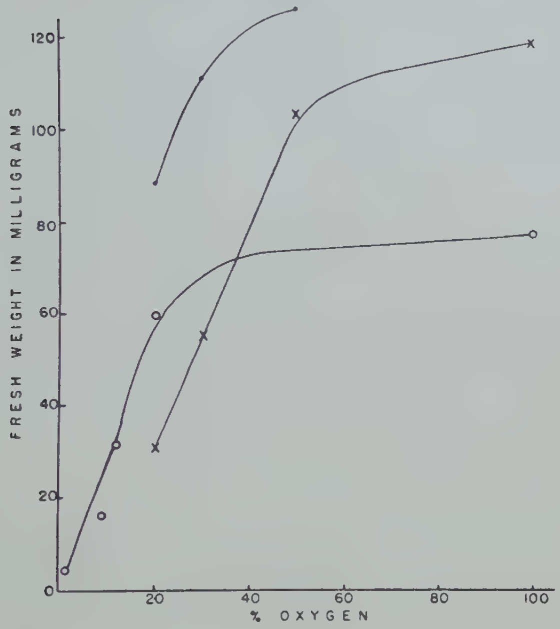
(ii) Also, for work with $C^{14}O_2$ to investigate carbon dioxide fixation, &c., one needs to be able to grow the tissue in contact with an enclosed atmosphere which contains this gas or, if C^{14} is supplied as bicarbonate, a confined space is necessary to prevent its escape to the atmosphere.

Investigation shows that the tissue cultures are not limited during 10–14 days by the supply of oxygen when the volume of the atmosphere to which they have access is of the order of 105 ml. By a further adaptation of the device illustrated above for collection of carbon dioxide, an additional attachment (Pl. VI) may be made of a flask which increases the volume to 600 ml., and which is equipped with an inlet and an outlet. This permits the gas to be swept out periodically. In this way any desired oxygen tension may be maintained by the use of gas mixtures prepared for the purpose in cylinders.

The effect of oxygen on growth of carrot explants. Oxygen tensions below that of air limit the growth of carrot explants under the standard conditions which have been described (Text-fig. 5). Varying from carrot to carrot, explants removed at a standard distance from the cambium can respond to greater oxygen concentrations in the gas phase above that of air. Using the growth of the culture in air as a standard of reference, it is clear that the curve relating oxygen tension to growth is of the same form for cultures from different carrots, but the gas composition which produces in the tissue a condition of oxygen 'saturation' may differ from carrot to carrot. The general similarity between the curves which relate relative growth to oxygen pressure (Text-fig. 5) and the previous curves of carbon-dioxide output of carrot disks in distilled water or salt (KBr) solution (Steward, Berry, and Broyer, 1936) is obvious.

Dark fixation of $C^{14}O_2$. In order to generate $C^{14}O_2$ in the atmosphere to which tissue cultures are subjected, the elliptical centre vessel may be modified by blowing on it small bulbs or nipples and placing these close together so that they may be charged with fluid by a pipette via an outlet which can be closed by a stopper. Into one of these bulbs may be placed the $C^{14}O_2$ in the form of bicarbonate and into the other a sufficient amount of non-volatile acid (e.g. H_2SO_4 or succinic). Once assembled (Pl. V) the apparatus will allow the acid and radio-active bicarbonate to mix at the first turn of the shaft. As the apparatus revolves, the enclosed atmosphere in contact with the tissue and solution will rapidly approach equilibrium. For work on dark fixation the culture tube itself is covered with several layers of heavy, light-proof paper. Experiments have been carried out with $C^{14}O_2$ sufficiently to show that dark fixation of $C^{14}O_2$ certainly occurs in these growing tissue cultures. Both the alcohol soluble and insoluble fractions of the tissue were examined. The alcohol soluble fraction was acidified to ensure that the C^{14} detected was not in

(A) GROWTH IN TERMS OF MG. FRESH WEIGHT.



(B) RELATIVE GROWTH AIR = 100

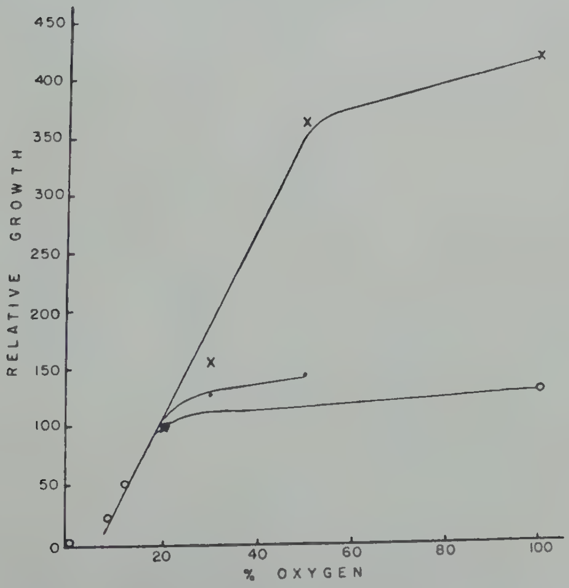


FIG. 5. Effect of oxygen partial pressure on growth in 14 days of carrot explants from different carrots under standard conditions.

the form of carbonate ion. It may be stated here that dark fixation occurred in both the growing and the non-growing cultures and the C^{14} appeared in both the alcohol soluble and insoluble fractions. Any comparison of the effect of these treatments on the amount of such fixation must be deferred.

Use of radioactive ions to investigate absorption and metabolism. The solution which permits rapid growth of carrot explants, or that which furnishes all necessary metabolites but lacks the essential growth factor, may be enriched with any of the available isotopes which are radioactive and which can exist in the ionic form. Such an isotope which has been found to be particularly instructive is Cs^{137}, which the authors first used in other work carried out in collaboration with Dr. R. Overstreet of the University of California. This isotope has a long half-life and in its absorption it is assumed to be similar to other alkali metals, particularly potassium. Almost any isotope whose half-life is long enough so that experiments may be continued for several days may be used, and those that have already found a use in the investigation of salt accumulation are clearly applicable (Jacobsen and Overstreet, 1947).

Wherever it is profitable to use radioactive isotopes to trace out the utilization of specific elements in metabolism and growth, it is much easier to do this by applying the isotopes to growing tissue cultures under the conditions here described than to apply them directly to whole plants growing in the convential way. The isotopes of sulphur and phosphorus are obvious cases in point, for these can be applied in the form of sulphate and phosphate in the culture medium.

If a non-metabolizable radioactive isotope is placed in the culture solution containing coco-nut milk the carrot explants may be harvested after different periods of growth and *both* the external solution and the tissue culture assayed for radioactivity. This has been done using a Tracerlab Autoscaler, for the case of Cs^{137} added without inert carrier to the external solution. The sensitivity of the carrier-free radioactive technique is such that absorption by one tissue culture from as low a concentration as 10^{-9} M. can be measured and followed with time. At the end of an absorption period the tissue is first rinsed and blotted dry, weighed, dried to constant weight, and then finely ground. The activity in the ground tissue may be determined keeping the layer of material on the copper 'planchets' constant so that the measurements are not vitiated by the mass of the sample actually counted. Alternatively the radioactive ion may be extracted in water and the activity determined on an aliquot of extract which contains a minimum of dry matter.

At this stage only the type of problem which these growing and non-growing cultures present will be noticed.

In the first place, when the tissue culture is able to grow rapidly and proliferate, by virtue of the growth-promoting substances present in coco-nut milk, the absorption of Cs^{137} proceeds concomitantly with growth as this is recorded by the increase of fresh weight (Text-fig. 6). The parallelism here is sufficiently close to indicate that absorption of Cs^{137} and absorption of water are both controlled or limited by some consequence of the proliferation which occurs.

In fact, after the first 4 to 6 days, successive increments of water by the tissue were associated with proportional increments of Cs^{137} (Text-fig. 7).

The striking and somewhat unexpected contrast is as follows. In the absence of coco-nut milk, but in an otherwise complete nutrient solution, the carrot phloem will increase its fresh weight from 4·0 to 9·0 mg. in 14 days. During this period the cultures absorb Cs^{137} progressively until relatively high concentrations obtain in the tissue (in a given case the counts/sec./culture reached 250).

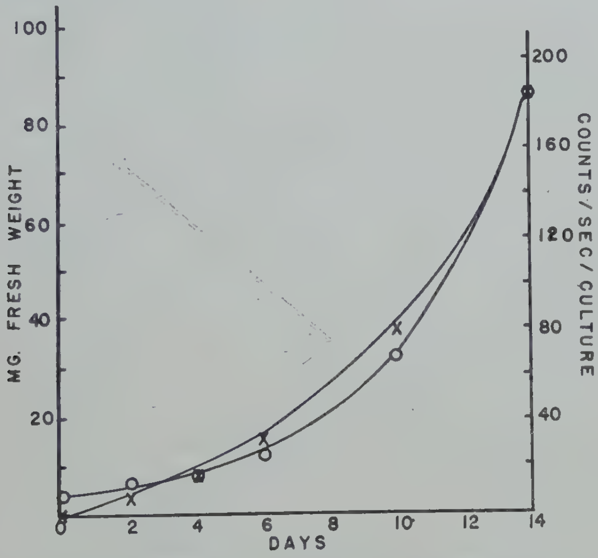


FIG. 6. Growth and absorption of Cs^{137} by carrot tissue-cultures in nutrient medium with coco-nut milk. O Mg. fresh weight. X Counts/sec./culture.

In the presence of coco-nut milk entirely comparable carrot phloem explants reached the same stage of 'growth' (i.e. 9·0 mg. fresh weight in as little as 4 days—i.e. at the end of the 'lag period' shown in Text-fig. 7). Accompanying this intake of water, however, relatively little Cs^{137} was absorbed (order of 16 counts/sec./culture). Thereafter, as growth (shown by the further increase of fresh weight) ensued, the intake of Cs^{137} occurred *pari passu* with water intake (reaching after 14 days in a given case 180 counts/sec./culture).

Clearly we have here two sharply contrasted systems. In both systems the tissue absorbs water and Cs^{137} concurrently and it does this progressively over many days in a manner which clearly indicates that in both cases the processes of growth and metabolism are concerned. They differ, however, very strikingly because the cultures in which external and random proliferation is stimulated by coco-nut milk have their absorption of water stimulated much more than their absorption of solute (Cs^{137}) as compared with the non-proliferating cultures.

It is precisely because the carrot tissue-culture system, used under the conditions that have been described, is so responsive in its water and salt intake to the conditions (supply of C.M.F.) that determine growth and proliferation that it is such a favourable medium in which to investigate the nature of the relationship between growth and metabolism on the one hand and intake of water and a radioactive ion on the other.

Protein synthesis and growth of carrot explants. Protein synthesis is a useful criterion of growth. Carrot-phloem explants, when freshly isolated from the root, may have their nitrogen predominantly in the soluble form (soluble nitrogen = 69 per cent. of total nitrogen). Exposing these explants to the nutrient and other conditions suitable for growth, but without supplying the essential coco-nut milk growth factor, causes a shift in the direction of protein nitrogen, but not a conspicuous overall synthesis of organic nitrogen. In presence of the coco-nut milk factor, however, growth and protein synthesis occur concomitantly as shown by Text-fig. 8. It will be noted that the growing cultures increase in total nitrogen *pari passu* with fresh weight, but that the increase is predominantly in the protein fraction. Also the growing cultures have a lower percentage dry weight than the carrot tissue from which they started and a definitely lower percentage dry-weight content than explants exposed to basal medium lacking coco-nut milk.

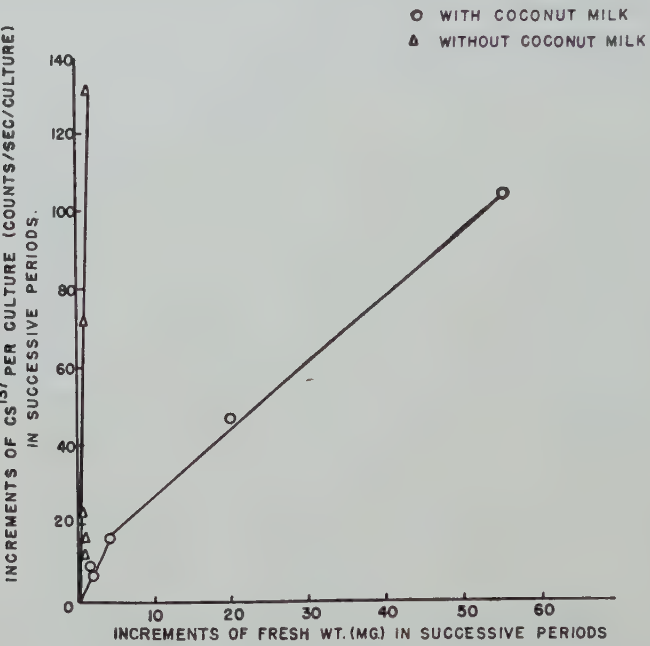


FIG. 7. Increments of water and Cs^{137} uptake in successive periods by tissue cultures.

To the extent that the coco-nut milk factor promotes *both* growth and protein synthesis, it functions as an analogue of the animal protein factor.[1] The new tissue-culture apparatus, combined with the use of coco-nut milk factor, therefore furnishes ideal experimental conditions for the investigation under controlled conditions of protein synthesis in these undifferentiated growing cells.

Application to the growth of micro-organisms. The culture technique described is particularly well adapted to the culture of micro-organisms which grow in water and in which (i) the cultures need to be gently agitated in order to prevent settling or 'clumping', (ii) the growth is such that it requires gas exchange or release, which can be accelerated by gentle agitation. By culturing *Chlorella* in this way it was found that rapid growth rates were achieved. (To keep the *Chlorella* from sticking to the sides of the rotating culture tube it is helpful to insert a solid glass bead into the culture.)

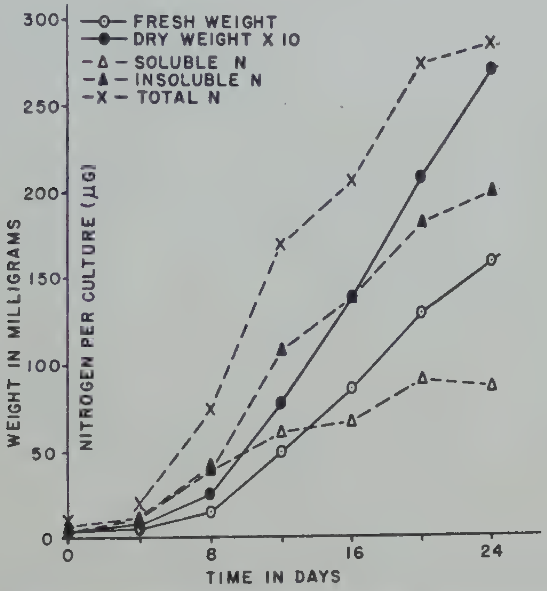


FIG. 8. Changes in weight and nitrogen fractions of carrot-root explants in a basal culture medium containing coco-nut milk.

In the case of *Chlorella* the cultures become extremely dense and uniformly dark green in the proper nutrient solutions. The growth rate may be readily measured by haemocytometer counts made on aliquots from the solution, and the growth curve plotted against time. The density of cells per cu.mm. obtained after 12 days' growth (Text-fig. 9) indicates the growth which was achieved. An interesting point is that this density was obtained by the use of a general inorganic nutrient solution[2] modified from Hoagland's solution for the growth of a particular plant. For this strain of *Chlorella*, the solution and conditions described gave somewhat better growth than the solution of Craig and Trelease as used by Winokur (1948). Since in use the apparatus described has been found to be adaptable to the growth of *Chlorella*, it may well find still other uses in microbiology.

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[1] Actual trials have shown that the effects due to coco-nut milk factor cannot be obtained by the use of pure vitamin B_{12} or preparations of animal protein factor.

[2] Hoagland's nutrient solution modified as follows: Ca = 10; K = 4; NO_3 = 20 mg. equiv. per litre with Na added in the amount to preserve ionic balance.

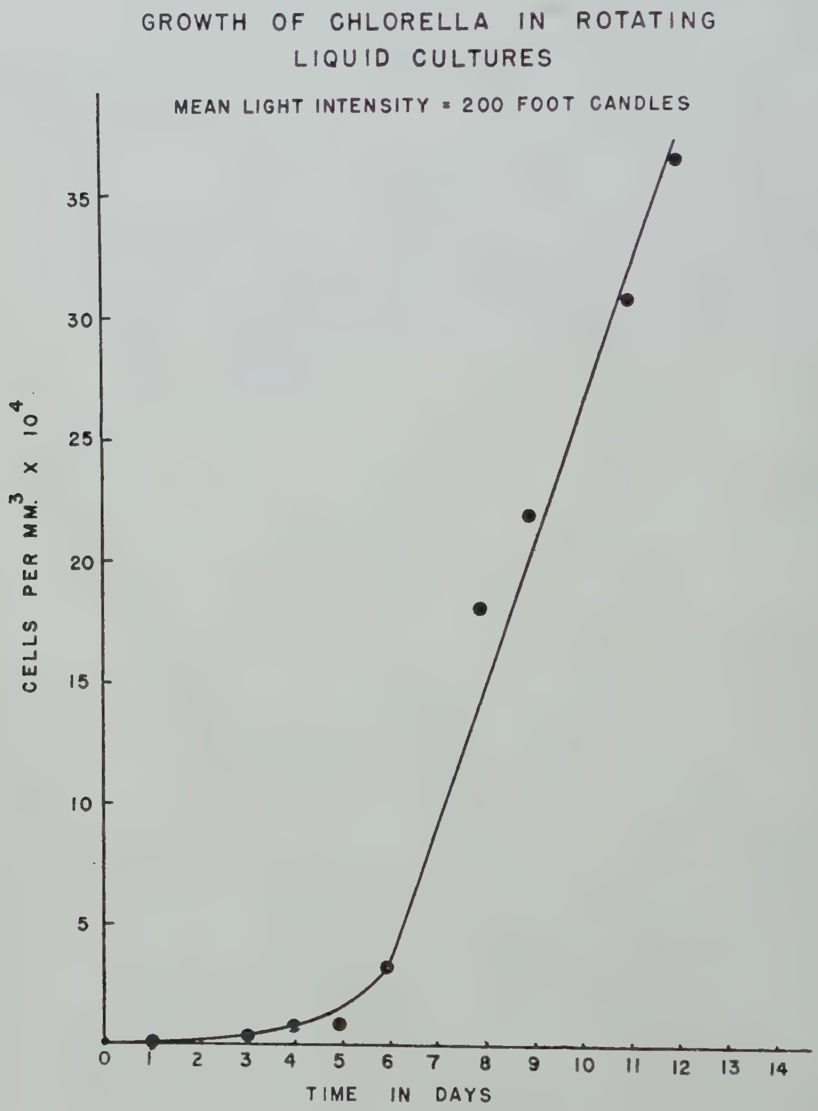

FIG. 9. Growth of *Chlorella* in rotating liquid cultures.

The technique as described is clearly very flexible and versatile. Not only does it fulfil its intended purpose to supply a method by which the behaviour in metabolism and salt uptake of growing and non-growing cells may be contrasted, but it also lends itself to a variety of purposes which entail the use of growing cells under controlled conditions. In so doing the method greatly extends the possible uses of plant tissue cultures in quantitative investigations of their growth and metabolism.

SUMMARY

1. A technique has been devised for the growth of plant tissue-cultures in liquid media under controlled experimental conditions. The measure of control now achieved in particular cases permits the tissue-culture technique to be exploited in the quantitative investigation of other physiological problems, especially the contrasted problems of the water and salt intake, metabolism and respiration of rapidly growing and non-growing cells.

2. By the use of explants of carrot secondary phloem in conjunction with coco-nut milk, added to an otherwise complete medium, a very active tissue culture can be obtained. By isolating the phloem only at known distances from the cambium it is at the same time possible to obtain precision and to reduce variability in the explants and also to secure a tissue which grows actively in response to the coco-nut milk factor (C.M.F.). By adding or omitting C.M.F. from the otherwise complete nutrient medium contrasted systems may be obtained from the same tissue which can be placed at will under circumstances where they *do* or *do not* actively proliferate and grow. Thus the difference between rapidly growing and non-growing cells may be investigated.

3. Various additional devices are described which may be attached to the new culture vessel for the control of the atmosphere with which the cultures are in contact. These devices permit the determination of their carbon dioxide output during growth as well as their uptake of ions and fixation of $C^{14}O_2$.

4. The time course of carbon dioxide output and of growth in coco-nut milk stimulated tissues are essentially similar.

5. The technique is far reaching in its scope: so much so that the apparatus has been given the general name of 'Auxophyton', i.e. a device which promotes the controlled increase of plants or plant cells.

6. Note is taken of the fact that the composition of carrot tissue-cultures as grown and of the original mature cells of the carrot root are quite different: the growing cells being richer in protein, poorer in soluble nitrogen (despite an external supply of nitrogenous nutrients), higher in water content, &c.

7. When growth is stimulated by the coco-nut milk factor, protein synthesis and uptake of an indicator ion (Cs^{137}) proceed concomitantly with growth as measured by increase of fresh weight. However, conditions in the relatively non-growing explant (i.e. when not treated with C.M.F.) for uptake of Cs^{137} are quite different, for the latter absorbs its salt relatively more quickly and relatively unaccompanied by water. These contrasts lend interest to this system in the further investigation of salt and water absorption.

8. The efficacy of the technique as described for the growth of *Chlorella* has been demonstrated.

Acknowledgements

These investigations have been supported by grants to the senior author from the Growth Committee of the U.S. National Research Council and from the National Cancer Institute, National Institutes of Health, Public Health Service. Miss Mary Case acted as research assistant during much of the work and carried out manipulations requiring aseptic precautions. During part of the work one of us (S. M. C.) held a Postdoctoral Fellowship of the National Institutes of Health, and another (F. K. M.) worked under a Predoctoral Fellowship in the Biological Sciences of the Atomic Energy Commission. For all this help we express our gratitude.

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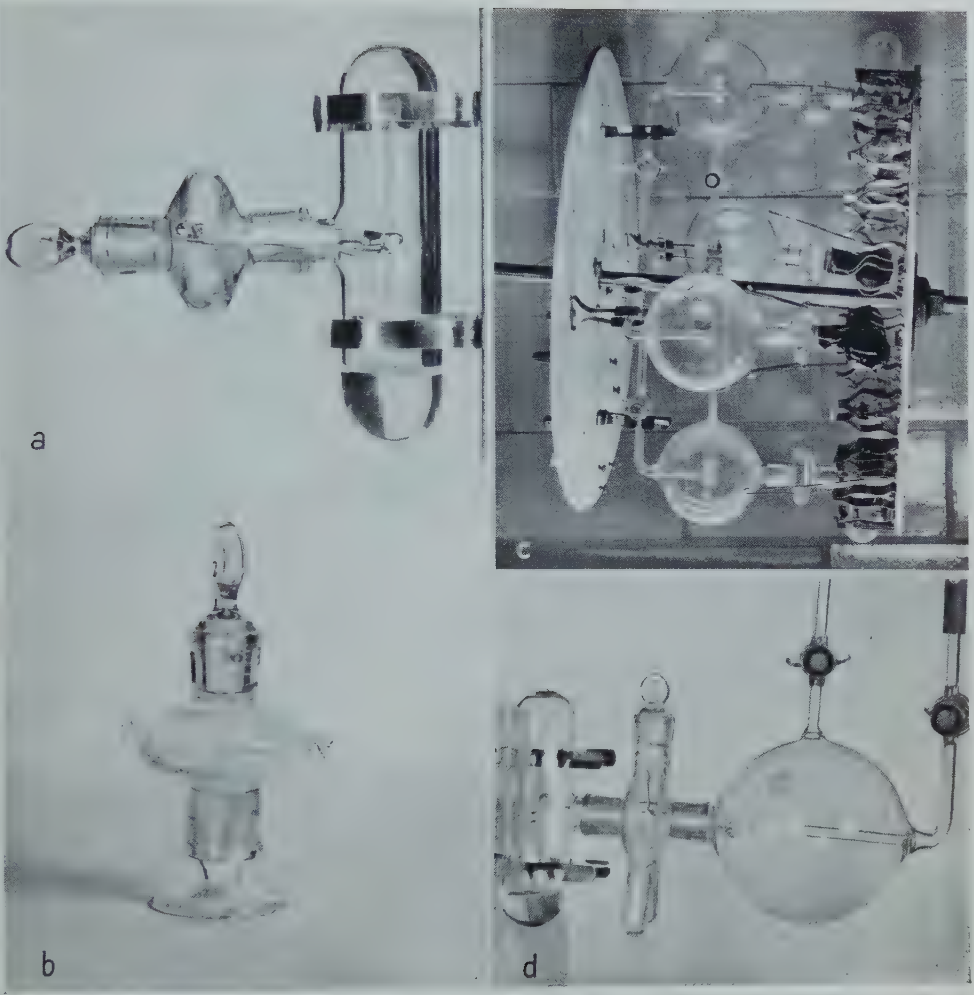
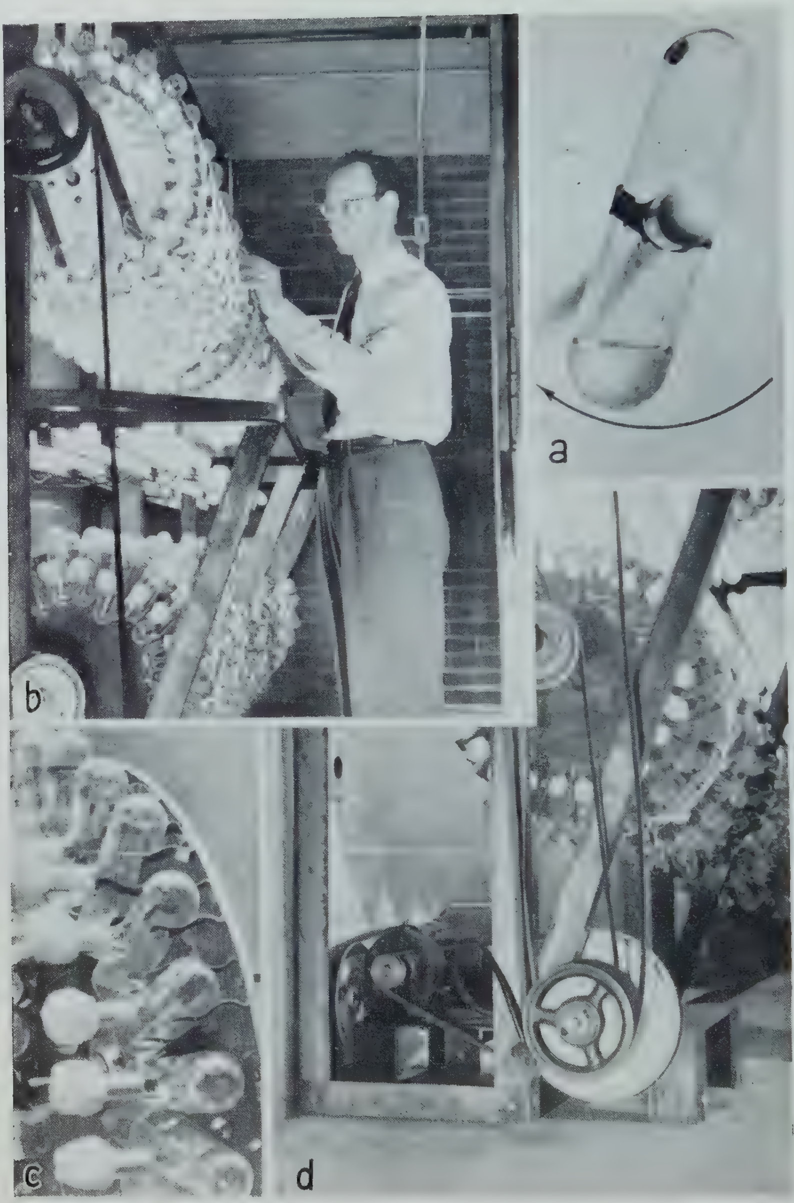


(*a*) Rotating culture tube with carbon dioxide absorber
(*b*) Carbon dioxide absorber awaiting analysis
(*c*) Apparatus for the effect of oxygen tension on growth and respiration
(*d*) Apparatus for the use of $C^{14}O_2$ in an enclosed system

F. C. STEWARD, S. M. CAPLIN, F. K. MILLAR



(*a*) Culture tube with explant in air
(*b*) General view of tissue culture apparatus
(*c*) Detail of mounting of the culture tubes
(*d*) Detail of drive

F. C. STEWARD, S. M. CAPLIN, F. K. MILLAR

Studies on the Physiology of Nodule Formation

III. Experiments on the Excision of Root-tips and Nodules

BY

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With Plate VII and seven Figures in the Text

ABSTRACT

The excision of nodules and root-tips from red clover inoculated with an effective strain of nodule bacteria leads to an increase in the number of nodules subsequently formed. This is thought to be due to the removal by excision of the source of an inhibitor. In the nodule the inhibitory activity appears to be centred in the growing-point and not in the bacterial tissue, since the excision of the nodular meristem only will stimulate further nodulation. The response to excision depends upon the number of excisions made and is inversely related to the inherent susceptibility of the individual plant.

Excision of nodules from plants inoculated with ineffective strains of bacteria has no influence on further nodulation, presumably because the growing-points of the nodules are abortive.

INTRODUCTION

IT has been shown in the first two papers of this series (Nutman, 1948, 1949*a*) that the infection of the clover root is determined by host as well as by bacterial factors. Infection appears to be restricted, first to those zones of the root bearing growing root-hairs, and secondly to definite meristematic foci within these zones. It is also suggested that the rate of initiation within the root of new meristematic centres is controlled by (1) the hereditary branching habit of the root, and (2) an inhibiting activity of the lateral and nodule meristems already present on the root.

A morphogenic relationship of this kind provides an explanation of the abundant infections promoted by ineffective strains of bacteria in terms rather of the plants' physiology than of the high infective virulence or invasive powers of the bacteria. On this hypothesis, the first formed ineffective nodules which are ephemeral do not possess any inhibitory activity and thus allow the initial high rate of infection to continue. Effective nodules, on the other hand, which are persistent, will tend to inhibit further infection. On this view, therefore, the relatively small numbers and large size of nodules formed by an effective strain are not due to any inherent lack in the capacity of the bacteria to gain access to the root tissues.

In this paper this hypothesis is examined experimentally in a study of the influence of excision of nodules and root-tips on further nodule and lateral development.

[Annals of Botany, N.S. Vol. XVI, No. 1, Jan., 1952.]

MATERIALS AND METHODS

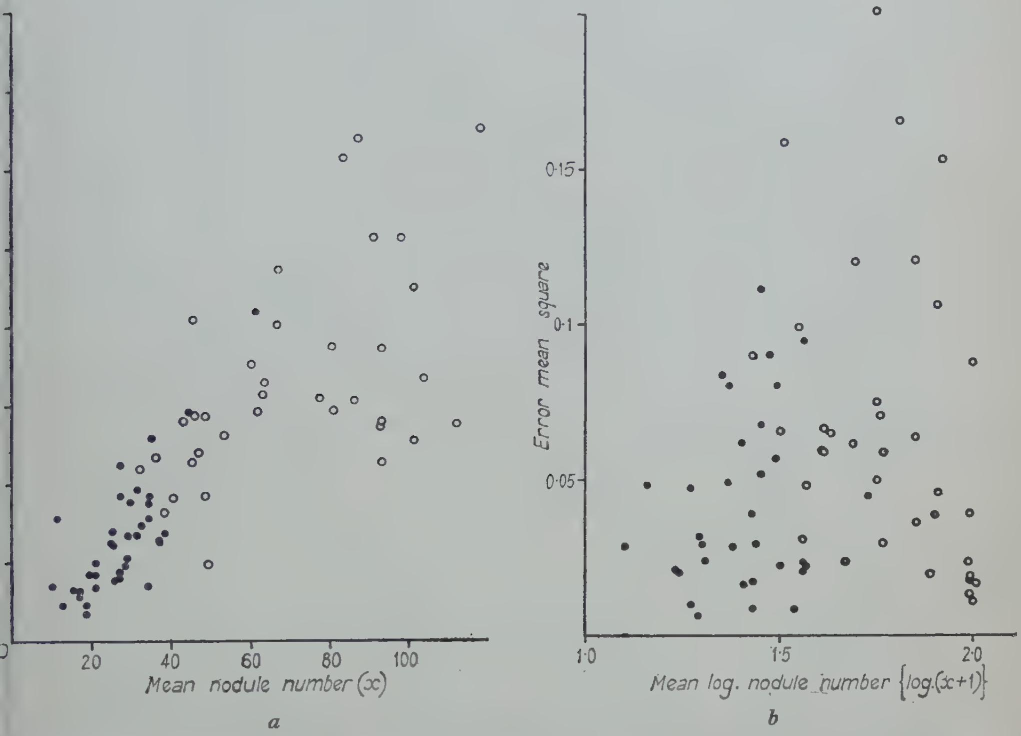
In all experiments red clover was used as the experimental plant—either commercial strains or selected lines bred in this department. The procedure for plant culture under bacteriologically controlled conditions has already been described, and with one exception, noted below, the strains of bacteria used as inocula are the same as in the previous experiments; viz. strain A, effective; strain HKC, ineffective.

Nodules and root-tips are difficult to excise from plants growing on agar slopes in test-tubes because of the limited space available for manipulation, the insecure anchoring of the plant on the surface of the medium, and the need to maintain sterile conditions. To overcome these difficulties a micro-guillotine was devised consisting of a small flat ring-shaped blade working in a slot at the end of a tubular holder. Excision is carried out by placing the blade over the nodule (or root-tip) and then withdrawing the blade into the slot at the end of the holder, or if the nodule is suitably placed it may be more convenient to cut it off with the sharpened end of the blade. After each operation the instrument is allowed to stand in a bath of boiling water for a few moments. This dislodges any fragments of agar or nodule and also allows an adherent film of oxide to form, which after sterilizing by flaming only is liable to flake off into the culture tube. From the water-bath the guillotine is transferred to a cylinder of alcohol and after flaming is placed horizontally on rests in a previously sterilized glass box to cool. The instrument is illustrated in Pl. VII and is made as follows:

A length of brass tubing (18 cm. long, internal diameter 1·3 mm., external diameter 3·3 mm.) is annealed and carefully hammered throughout its length until the lumen becomes rectangular in section (about 1·75 mm.×0·5 mm.). At one end, later to form the slot in which the blade works, the hammering is continued in the original direction, and also at right angles until the lumen is quite closed and the tube slightly drawn out. This end is next filed at right angles to its length until the lumen reappears as a narrow slit wide enough to take as a smooth fit a blade 0·05 mm. in thickness. The walls of the tube at this end are then reduced in thickness and bevelled, and after reannealing the last centimetre or so of the tube may be slightly bent so that the blade will work at a small angle to the rest of the tube.

The blade is made from 0·5 mm. gauge chrome-nickel wire twisted into a loop about 1·5 mm. diameter having a tightly twisted stem of 4 or 5 cm. behind the loop, the remaining 20 cm. consisting of a single strand of wire. The loop is pinched into an ellipse and then flattened by hammering on an anvil until it and its stem of twisted wire are about 0·25 mm. in thickness. The final thinning and shaping of the blade to fit the slot in the holder is done with file and fine emery paper. On threading the blade into the holder about 7 cm. of wire projects from the unshaped end. This is fastened to a brass slide working on an extension tube (18 cm. of brass tubing) having a slot through which the wire moves.

The plant cultures were arranged in racks on a glasshouse bench. Experience has shown that growth conditions are uniform so that on this account no division of racks into 'blocks' with a view to subsequent statistical analysis is necessary. In most experiments a completely randomized design was adopted, but in some (detailed below) a division was first made into 'blocks' based on plant types (family or strain) or on a preliminary selection into plants with many or with few nodules.



TEXT-FIG. 1. Relation between variance and mean of numbers of nodules and lateral roots in expts. 2 and 3 before (*a*) and after logarithmic transformation (*b*); ○ nodules, ● lateral roots.

At harvest the number of nodules per plant was recorded as well as, in some cases, the number of lateral roots and dry weight. In some experiments counts were also made of nodules and roots for as long as practicable without removing plants from their tubes.

For purposes of statistical analysis when treatment effects are to be compared it is important that the variance should be independent of the treatment. In this work the treatment effects are assessed from the final number of nodules or lateral roots produced and the data of experiments 2 and 3 have been examined to determine the relation between the variance and number of laterals or nodules. In Text-fig. 1 (*a*) the mean numbers of plant organs (x) is plotted against their standard errors. It is evident that variance is highly correlated with increasing numbers of laterals or nodules. In the analyses carried out, therefore, a logarithmic transformation ($\log_{10}(x+1)$) has been employed, the effect of which is shown in Text-fig. 1 (*b*).

S. N. Bhaduri has also shown in unpublished work in this department that for material grown under similar conditions a logarithmic transformation is desirable for dry weight as well as for nodule number.

EXPERIMENTAL

Experiment 1

This preliminary experiment was designed to determine the effect of excision of the main root apex, or of two, four, or eight nodules on the subsequent rate of infection with an effective strain of bacteria, and also to determine the effect of the excision of eight nodules with ineffective strain inoculation. A single family of plants was used with about twelve replicated plant cultures in each treatment. The excision of the main root-tip (3 mm. in length) was made 15 days from sowing and the nodules 35 days from sowing.

After a total growth period of 57 days the numbers of nodules were counted; the results are set out in Table I, which shows both the mean number of nodules at harvest in each treatment and the mean of the logarithms of numbers (+1) of nodules in each treatment. On plants upon which excisions had been carried out the number of nodules excised has been added to the actual number of nodules counted at harvest to give the total number of completed infections sustained by each plant.

TABLE I

Table of Mean Number of Nodules at Harvest and Mean of Log₁₀ (Number+1) of Nodules at Harvest

	Inoculated strain A		Inoculated strain HKC	
Treatment.	Mean number of nodules (x)	Mean log. (x+1)	Mean number of nodules (x)	Mean log. (x+1)
Control . . .	(C) 48·4	1·657	(Ci) 118·9	2·049
Main root-tip excised	(IR) 67·6	1·753	—	—
Two nodules excised	(2N) 46·6	1·661	—	—
Four nodules excised	(4N) 70·7	1·787	—	—
Eight nodules excised	(8N) 72·1	1·840	(8Ni) 111·0	1·995

Mean differences (Mean squares 128·5; 0·03664)

IR—C	19·2±14·8	0·096±0·101
2N—C	1·8±10·4	0·004±0·093
4N—C	22·3±20·3	0·130±0·096
8N—C	23·7±11·2*	0·183±0·081*
8Ni—Ci	—7·9±15·2	—0·054±0·081

Levels of significance: *$P = 0·05$; **$P < 0·01$.

The excision of the main root-tip or of four or eight nodules increased the subsequent infection rate by an effective strain of bacteria, although only to a significant extent in the last instance. The excision of two nodules is apparently without effect. On plants inoculated with an ineffective strain even the excision of eight nodules has no influence on further development.

These results support the hypothesis that nodule formation is determined by the number of active nodules already present on the root. Pre-existing nodules of an effective type tend to inhibit further nodule production so that their excision leads to an increased rate of infection. Nodules of an ineffective type, on the contrary, are ephemeral and the supposition that they should therefore have no inhibitory action on further meristematic development in the root is borne out by the absence of any response to excision.

The stimulation following the excision of effective nodules is small and the next two experiments were designed to determine its magnitude more precisely for two rates of excision, and also to compare nodule with lateral root excision, and early excision with late excision.

Experiments 2 and 3. Factorial experiments with effective and ineffective strains of bacteria on rate, time, number, and type of excision

These experiments were similar in plan and can be considered together. In both a factorial design was employed in which the following main comparisons could be made:

1. Lateral root-tip excision *v.* nodule excision (N–R).
2. The excision of three units (nodules or root-tips) *v.* the excision of six units (6–3).
3. Excision early in seedling growth *v.* later excision (1st–2nd).
4. Effective strain inoculation *v.* ineffective strain inoculation.

In addition sets of unexcised plants were run as controls.

The first of these experiments was set up on 25/2/48 and harvested on 4–8/6/48 and comprised plants from ten different sources, arranged in blocks with random distribution of treatments within the blocks. The second experiment was set up on 21/6/48, harvested in early October, and was planted with a batch of three families of seeds which were mixed before sowing. In this experiment the two groups of treatments inoculated with effective and ineffective strains were kept separate, the control and excision treatments again being distributed at random in each.

In the first experiment the location of some of the points of excision of nodules and roots were marked on the outside of the culture tube and frequent observations were carried out to determine whether regeneration took place after operation. No regeneration of roots or nodules was observed at the cut surface, and the stumps of cut laterals afterwards elongated only very slightly.

Counts were made of lateral roots and nodules throughout each experiment and at harvest. The final treatment means are given in Table II, and statistical analysis in Table III.

In each experiment with the effective strain, excision had overall a significant effect in stimulating further nodulation. Both root-tip and nodule

Table II

Experiments 2 and 3: Table of Means

Treatment.	Logarithms of number (+1) of infections				Logarithms of number (+1) of roots				Dry weight per plant in mgm.	
	Strain A		Strain HKC		Strain A		Strain HKC		Strain A	Strain HKC
	Expt. 2.	Expt. 3.	Expt. 2.	Expt. 3.	Expt. 2.	Expt. 3.	Expt. 2.	Expt. 3.	Expt. 3.	Expt. 3.
Control	1·496	1·430	1·853	1·850	1·408	1·457	1·103	1·408	42·28	15·87
3 nodules excised early .	1·671	1·692	1·916	1·750	1·376	1·494	1·167	1·316	49·83	16·40
3 ,, ,, late . .	1·563	1·609	1·809	1·860	1·357	1·500	1·270	1·438	48·74	16·47
6 ,, ,, early .	1·768	1·610	2·041	2·004	1·572	1·475	1·041	1·381	49·13	16·94
6 ,, ,, late . .	1·757	1·692	1·773	1·955	1·377	1·564	1·235	1·433	51·05	15·87
3 root-tips excised early .	1·553	1·518	1·916	1·958	1·457	1·566	1·291	1·278	50·97	18·47
3 ,, ,, late . .	1·620	1·572	2·003	1·941	1·442	1·568	1·304	1·433	58·12	20·58
6 ,, ,, early .	1·681	1·643	1·997	1·890	1·459	1·543	1·241	1·347	46·05	20·11
6 ,, ,, late . .	1·753	1·771	1·973	1·905	1·495	1·737	1·290	1·457	60·43	19·38

TABLE III

Experiments 2 and 3: Table of Mean Differences

Key:

Treatment mean minus control mean	E−C
Nodule excision mean minus root excision mean	N−R
Excision of 6 units minus excision of 3 units	6−3
Early excision minus late excision	1st−2nd

		Experiment 2.	Experiment 3.
Log$_{10}$ *No.* (+1) *infections*		(Mean square 0·07644, 118 d.f.)	(Mean square 0·05257, 122 d.f.)
Strain A	E−C	0·174±0·083*	0·207±0·082*
,,	N−R	0·044±0·076	0·036±0·061
,,	6−3	0·135±0·076	0·091±0·062
,,	1st−2nd	−0·010±0·076	−0·047±0·062
Strain HKC	E−C	0·078±0·082	0·064±0·070
,,	N−R	−0·083±0·075	−0·022±0·059
,,	6−3	0·042±0·075	0·046±0·058
,,	1st−2nd	0·074±0·075	0·005±0·058
,,	Root excision−C	0·119±0·091	0·074±0·075
Log$_{10}$ *No.* (+1) *of roots*		(Mean square 0·04745, 118 d.f.)	(Mean square 0·03759, 122 d.f.)
Strain A	E−C	0·032±0·067	0·100±0·070
,,	N−R	−0·046±0·060	−0·100±0·052*
,,	6−3	0·061±0·060	0·058±0·052
,,	1st−2nd	0·044±0·060	−0·069±0·052
,,	Root excision−C	0·055±0·072	−0·153±0·075*
Strain HKC	E−C	0·128±0·065*	−0·018±0·059
,,	N−R	−0·107±0·059	0·033±0·053
,,	6−3	−0·059±0·059	0·013±0·038
,,	1st−2nd	−0·092±0·059	−0·110±0·053

		Experiment 3.	
		Dry weight	*Log dry weight*
Dry weight		(Mean square 247·86)	(Mean square 0·02730)
Strain A	E−C	10·08±5·41	0·133±0·057*
,,	N−R	− 5·40±4·21	−0·054±0·044
,,	6−3	0·67±4·22	0·004±0·044
,,	1st−2nd	− 5·39±4·23	−0·041±0·044
		(Mean square 11·97)	(Mean square 0·007668)
Strain HKC	E−C	2·37±1·03*	0·064±0·026*
,,	N−R	−3·23±0·89**	−0·079±0·023**
,,	6−3	0 —	−0·001±0·025
,,	1st−2nd	− 0·22±0·89	−0·005±0·023

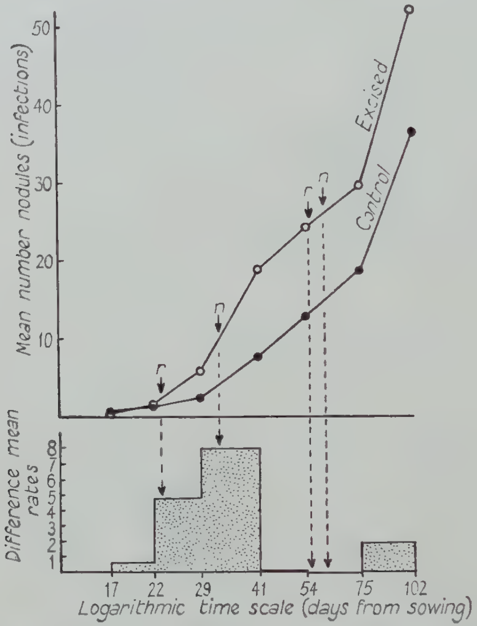
Levels of significance: * $P = 0·05$; ** $P < 0·01$.

Significant differences in combined data of experiments 2 and 3.

E−C	for nodules with strain A, **
6−3	,, nodules ,, ,, *
N−R (−)	,, roots ,, ,, *
R−C	,, roots ,, ,, *
R−C	,, nodules ,, ,, HKC ($P = 0·1$)
1st−2nd (−)	,, roots ,, ,, **
E−C	,, dry weight with strain A, *
E−C	,, ,, ,, ,, HKC, *
N−R (−)	,, ,, ,, ,, **

excision contributed equally to this increase; the slightly higher stimulation following nodule rather than root excision was statistically insignificant. It is also clear from both experiments that a larger increase in nodulation followed the excision of six rather than three nodules or root-tips. The slight advantage of late over early excision was statistically insignificant.

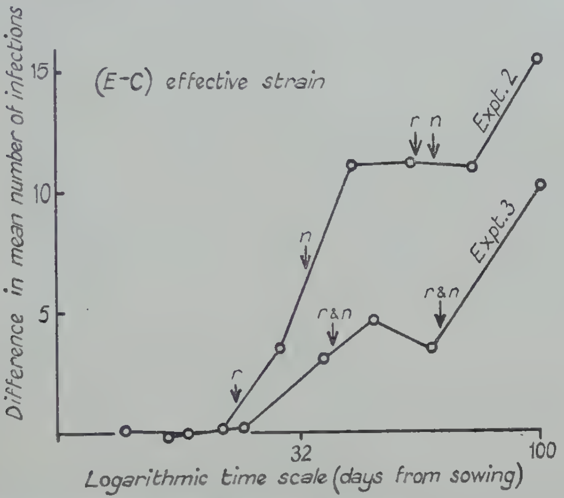
With ineffective strain inoculation no significant differences in nodulation rate were found in either experiment. Some stimulation might have been expected following lateral root excision leading to a higher proportion of the total meristems on the root to be associated with infection. A larger number of nodules were produced in each experiment following root excision and the extra comparison (root excision–C) is entered on the table. Although insignificant, the results show a difference in the expected direction. In view of the very large number of nodules produced by the ineffective strain a large experiment would be required to confirm this effect.



TEXT-FIG. 2. The progress of nodule formation on control and on excised plants inoculated with an effective strain of bacteria; below, the mean difference in rates of nodule formation between control and excised treatments. Arrows show times at which nodule (*n*) and root-tip (*r*) excisions were made.

The results for the final number of lateral roots are difficult to interpret because of a number of contradictory trends in the two experiments. Thus with strain A the comparison (1st–2nd) gave a positive difference in expt. 2 and a negative difference in expt. 3. Similarly with strain HKC, the comparison (N–R) is negative and approaches a level of significance in expt. 2 and is positive and insignificant in expt. 3.

Two consistent and significant differences should however be noted; i.e. (N–R), or the related comparison (root excision–C) with effective inoculation and (1st–2nd) with ineffective inoculation. The former suggests that with effective strains root excision is more stimulatory to further rooting than nodule excision—which it will be recalled is contrary to their effects on nodule formation. The latter suggests that late excision of roots or nodules with an ineffective strain does stimulate further rooting, whereas early excision has no influence.

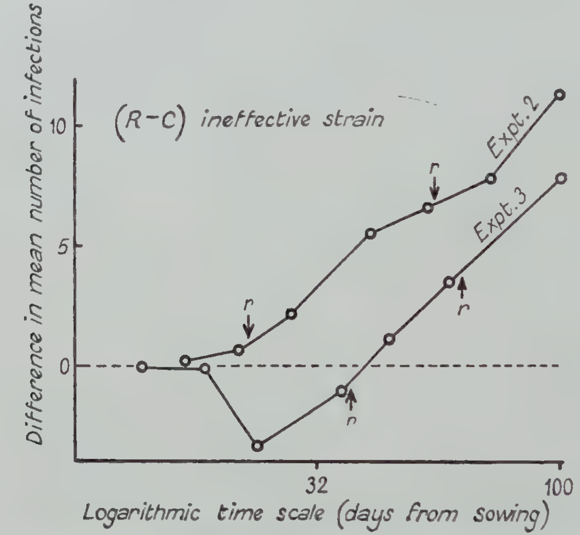


TEXT-FIG. 3. Difference between mean number of infections on control and excised plants inoculated with an effective strain in expts. 2 and 3. Arrows show times at which nodule (*n*) and root-tip (*r*) excisions were carried out.

Rates of nodule formation. Previous studies (Nutman, 1948, 1949 *a*) have shown that under the conditions of growth employed the rate of infection progressively declines, an approximately constant rate appearing on a logarithmic time scale. The results of expt. 2, performed with an effective strain and graphically presented in Text-fig. 2, again show a linear relationship between nodule number and logarithm of time over the first 80 days. The results also show that with effective inoculation the response to excision is both rapid and transient. The combined curves of nodule formation for the excised plants diverge from the controls soon after the first excisions and then become sensibly parallel, indicating a return to the same growth rate as the control. After the second set of excisions, however, the treated plants again show an increase in rate over the controls.

The differential behaviour in the rate of nodulation between control and excised plants is indicated in the lower histogram of Text-fig. 2, which shows the mean difference in rates calculated over successive intervals of time. During the third interval the difference is maximal and falls to zero in the fourth and fifth periods, rising again in the last interval following the second excisions.

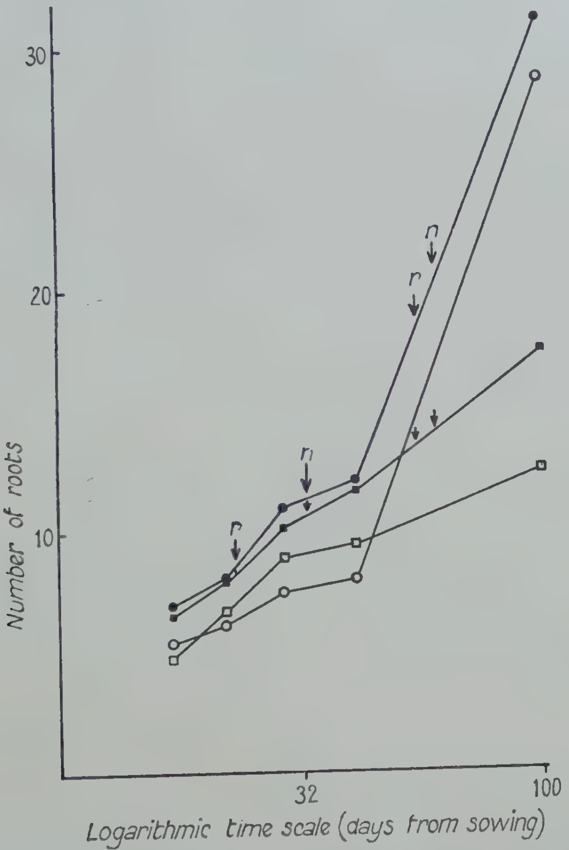
This is shown in another way in Text-fig. 3, in which the differences between the number of nodules present on excised and control plants are plotted on a logarithmic time scale. The results for expt. 3 are included on the same figure and are confirmatory although the effects are smaller. Both experiments show that following excision the rate of nodulation first increases and then falls again to resume its original level so that the relative difference between control and excised sets diminishes with time. These results may be compared with the increase in rate shown to follow delayed inoculation (with effective strains of bacteria) as reported in the previous paper (p. 266). It was there shown that after delayed inoculation nodule formation began at the same relative rate as the control plants and that the curves continued to run parallel to the end of the experiment.



TEXT-FIG. 4. Difference between mean number of infections on control plants and on plants from which root-tips were excised, with *ineffective* strain inoculation. Arrows show times at which nodule (n) and root-tip (r) excisions were carried out.

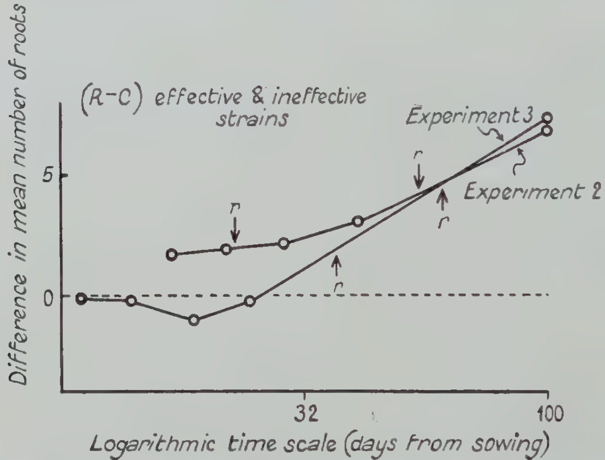
With *ineffective* strain inoculation, as noted above, a slight stimulation of nodule formation following root excision, although insignificant, was observed at the end of the experiment. In Text-fig. 4 the mean differences in numbers of nodules produced between control and treated plants are plotted on a logarithmic time scale. The results are similar for those already shown for effective inoculation. In expt. 2 there is an increase in the difference after the first excisions followed by a period during which little further increase takes place until after the second excisions. A corresponding step in the curve for expt. 3 does not appear.

Rates of root formation. With the effective strains of bacteria root production also at first shows a linear increase on a logarithmic time scale. At the end of this phase, however, roots arise at an increased rate although they remain largely uninfected (see previous paper). Root production after excision also consists of two phases as shown in Text-fig. 5. Counts were not made frequently enough to fix the time at which the change in root production occurred, or to analyse the influence of each of the excision treatments upon the two phases of growth.



TEXT-FIG. 5. The progress of lateral root production on control and excised plants of expt. 2: ○, control strain A; ●, excised strain A; □, control strain HKC; ■, excised strain HKC. Arrows show times at which nodule (*n*) and root-tip (*r*) excisions were carried out.

The increase in the rate of root production has been attributed to the cumulative effect of additional nitrogen supply due to nodule activity, and for this reason no change of this kind appears with the ineffective strain HKC. The differences in root number at the beginning in the respective control and excised groups must be due to sampling error, but nevertheless the rate of root production as shown by the slopes of the curves is in fairly good agreement. In so far as the effect of excision is concerned there is slight evidence of increase in lateral root formation following the first root excision in both the strains. After the second excision, however, the evidence is conflicting, as the statistical analysis in Table III has already indicated. Owing to the fact that no root counts were made in the interval between the second excision and the end of the experiment any transient effect would have been missed, and as shown above in the case of nodule formation such transient effects occur. Even so, in the ineffective strain the final root number is considerably higher in the excised set, due principally to the second sets of excisions (see Table III; 1st–2nd (—) for roots, $P<0\cdot01$). The actual differences between control and treated plants, combining the values for the two strains, in the number of lateral roots produced are plotted in Text-fig. 6, which shows that for both expts. 2 and 3 a higher rate of lateral root production occurs in the excised plants.



TEXT-FIG. 6. Mean differences in numbers of roots developing on control plants and on plants from which root-tips were excised and which were inoculated with effective or ineffective strains. Arrows show times at which nodule (*n*) and root-tip (*r*) excisions were carried out.

Dry weight. Individual dry weights were determined for all plants in expt. 3 (neglecting the weight of nodules excised); the analyses of the results are shown in Table III. With either strain of bacteria excision has resulted in larger plants. Such a result is to be expected in the case of the effective strains; there is an increase in the number of nodules contributing nitrogen to the plant, and for this reason the final weights of the plants in the excised sets are higher. This consideration, however, would not appear to apply to the ineffective strain. The increase in dry weight over the controls is much smaller, but owing to the much reduced variance this difference is statistically significant. The variation with strain A is much greater, so that the increase in dry weight over the controls in this set does not quite reach the requisite level of significance on the untransformed data. Since the effect of efficient nodules on dry weight is cumulative it is more appropriate to carry the analysis through for strain A on the basis of the logarithm of dry weight; the increase in dry weight due to excision now becomes clearly significant (see Table III).

It will also be noted that on the basis of logarithm of dry weight the results with the ineffective strain noted above remain significant. This would suggest that the ineffective strain contributes nitrogen to the plant, as Chen and Thornton (1940) have shown, but that the net amount is small because of the early degeneration of the nodules. If, however, as may be suggested, excision of root-tips in particular (see Table III) delays degeneration of ineffective nodules, the results would be explained. Experiments will be undertaken to elucidate this matter further.

The remaining experiments to be described were designed to investigate a number of problems raised by expts. 2 and 3. These will be discussed in turn although the relevant experiments overlap to a certain extent. Different commercial samples of clover were used in all experiments (except in expt. 4); convenient plants for excision were selected before a set of treatments were applied. The usual replication was about ten plants per treatment and twice this number in the control set. All experiments were sown in the spring (March, April, or May) and were harvested after about 100 days' growth. Excisions were carried out at about 30 days from sowing. In expt. 5 the bacterial strain 'Clover F^2 was used as inoculum. This strain is of slightly greater effectiveness than strain A which was used in all the remaining experiments.

Experiment 4. The excision of roots and nodules of hereditarily ineffective plants

Experiments 1, 2, and 3 demonstrate the important influence of bacterial strain type in experiments on excision. This influence was predicted on the hypothesis of inhibition from active meristems and may be attributed to the degeneration of the nodular meristem in ineffective symbiosis and its persistence in the effective. That it is not directly a bacterial strain effect has been tested with clover selected and bred to respond ineffectively with the normally effective strain A. This material has been shown (Nutman, 1949 *b*, p. 277) to differ from ordinary red clover in a simple homozygous recessive factor. The response of this material is typically ineffective with strain A inducing the formation of large numbers of ephemeral nodules, but is normally effective with other strains of bacteria. Three treatments were employed: unexcised and with eight nodules or roots excised. The results are set out in Table IV and show that there is no effect of excision of roots or nodules on initiation of either roots or nodules: i.e. ineffective nodules containing either wild-type ineffective strains or normally effective bacteria are equally inactive in inhibition.

Effect of disturbing agar medium and root system. In the earlier experiments in which no selection of plants was made prior to excision, the agar slopes had often to be considerably disturbed and the roots manipulated into positions convenient for the excisions to be carried out. Tests of the effect of such operations apart from excision were made in two experiments. Roots and nodules were disturbed to the maximum degree employed for the excision of six nodules in the first experiment and four in the second, the guillotine

TABLE IV

Excision of Nodules or Root Tips of Hereditarily Ineffective Clover inoculated with Strain A

Mean log. number (+1) *of infections at harvest*	
Control (C)	2·035
8 nodules excised (8N)	2·094
8 roots excised (8R)	1·980
Mean differences (Mean square 0·05758)	
8N−C	0·059±0·096
8R−C	−0·055±0·098
8N−8R	−0·114±0·096
Mean log. number (+1) *of lateral roots at harvest*	
(C)	1·628
(8N)	1·575
(8R)	1·647
Mean differences (Mean square 0·01583)	
8N−C	−0·053±0·050
8R−C	0·019±0·051
8N−8R	−0·072±0·050.

being placed in position without excision actually being made. The results are summarized in Table V; a comparison is made between disturbing the roots, excision, and no treatment. In each experiment the disturbed sets are intermediate in the number of nodules formed, the differences between control and disturbed sets in the first experiment and between excised and disturbed sets in the second approaching a level of significance.

TABLE V

Stimulation of Infection following Disturbance of Roots

	Experiment 3.		Experiment 5.	
Log. number (+1) *of infections at harvest*				
Control	(C)	1·430	(C)	1·391
Disturbance	(6D)	1·603	(4D)	1·459
Excision	(6N)	1·689	(4N)	1·535
Mean differences, mean squares	0·05357		0·04415	
N−C	0·259±0·090*		0·144±0·059*	
N−D	0·086±0·090		0·076±0·060	
D−C	0·173±0·108		0·068±0·060	

Test of significance on combined data of expts. 3 and 5

N−C	**
N−D	$P = 0·1$
D−C	$P = 0·1$

The considerable disturbance involved in these experiments was thus itself stimulatory to nodule formation although to a lesser degree than complete excision, possibly for the same reason. Disturbance usually involved making sharp bends in the roots and this may interfere with the transport or formation of an inhibitor.

In later experiments effects due to this cause were avoided by first selecting plants for convenience of excision so that the agar or roots need not be disturbed, the various treatments and controls being allocated at random after selection.

Further experiments comparing nodule and root excision. Experiments 2 and 3 suggested that nodule excision may be slightly more stimulatory than root excision—a possibility examined in further experiments listed in Table VI. The results show a consistent and slightly higher nodule count following nodule excision which approaches a level of significance in the five experiments considered together. The difference is small, amounting on the average to 0·0334 units per nodule excised (equivalent to 1·08 nodules). This result suggests that a nodule meristem is rather more active in inhibition than a root apex—a difference which may be related to its larger size.

TABLE VI

Comparison of Nodule and Root Excision in All Experiments

	Experiment 2.	Experiment 3.		Experiment 6.	Experiment 7.
Mean log. number (+1) *of infections at harvest*					
Nodule excision .	(3 & 6 N) 1·692	(3 & 6 N) 1·654	(1 N) 1·678	(3 N) 1·538	(6 N) 1·641
Root excision . .	(3 & 6 R) 1·648	(3 & 6 R) 1·617	(1 R) 1·581	(3 R) 1·474	(6 R) 1·524
Primary root excision .	—	—	(1 PR) 1·606	—	—
Mean differences,					
mean squares . .	0·07644	0·05257		0·04183	0·03361
N−R . . .	0·044±0·076	0·036±0·061	0·097±0·115	0·063±0·099	0·117±0·075
R−PR . . .	—	—	0·024±0·123	—	—

Test of significance on all experiments N−R, $P = 0{\cdot}1 - 0{\cdot}05$.

Excision of nodule meristem. In view of the similar behaviour of nodules and root-tips as inhibitory organs, an attempt was next made to determine whether the activity of the nodule resided in the apex or bacterial infected tissue. For this purpose nodule meristems only were excised from some plants and in others a partial meristem excision was carried out by means of a transverse cut passing half-way through the nodule near its tip. In no case was a nodule meristem regenerated following complete excision and only in one instance was growth continued after partial excision. Four experiments of this kind were carried out with the results shown in Table VII. In each experiment the excision of the nodule meristem stimulated further nodulation to the same degree as complete excision, and in two experiments out of three the fewer nodules produced in the treatment in which a transverse cut was made in the nodule, compared with complete excision, were not significant. These results support the contention that the inhibitory activity resides in the growing-point of the nodule rather than the bacterial tissue. The partial isolation of the meristem by means of a transverse cut stops both its growth and much reduces, if not completely eliminates, its inhibitory activity. Experiments were next undertaken to determine if any inhibitory activity remained in the cut-off nodules which were usually left in the tube.

TABLE VII

Nodule Excision versus Nodule Meristem Excision

	Experiment 3.		Experiment 5.		Experiment 6.		Experiment 7.	
Mean log. number (+1) of infections at harvest								
Control	(C)	1·430		1·391		1·340		1·610
Nodules excised	(6 N)	1·689	(4 N)	1·535	(3 N)	1·538	(6 N)	1·641
Nodule meristems excised	(6 NM)	1·683	(4 NM)	1·579	(3 NM)	1·565	(6 NM)	1·586
Nodule meristems half excised	—		(4 N ½ M)	1·458	(3 N ½ M)	1·524	(6 N ½ M)	1·686
Mean differences, mean squares	0·05257		0·04415		0·04183		0·03360	
N − C	0·259** ± 0·090		0·144* ± 0·057		0·236* ± 0·099		0·031 ± 0·072	
N − NM	0·006 ± 0·116		0·044 ± 0·057		−0·028 ± 0·102		0·054 ± 0·072	
N − N ½ M	—		−0·077 ± 0·057		0·013 ± 0·099		−0·044 ± 0·073	

Test of significance on combined data: N − C ***
N − NM not significant
N − N ½ M not significant.

Effect of removal or addition of nodules to culture tube. Two experiments were carried out to determine residual inhibition from cut-off nodules. In the first the excised nodules were transferred from one treatment in which six excisions were made to another which had suffered an equal number of excisions. The usual controls were also run so that the direct effect of excision could be determined in the absence of the cut-off nodules and in the presence of six and twelve cut-off nodules. In the second experiment four excisions were made per plant and the nodules taken from one treatment were added to a second control set on which no excisions were made. The results are set out in Table VIII. In the first test there is clearly no effect of either adding or taking away cut-off nodules from the system. The second experiment, how-

TABLE VIII

Test of Inhibition from Excised Nodules

	Experiment 3.	Experiment 5.
Log. number (+1) of infections at harvest		
A. Control 1, no excision	1·430	1·391
B. Control 2, no excision plus excised nodules from another plant	—	1·546
C. Normal excision	1·689	1·535
D. Excision, plus additional excised nodules	1·721	—
E. Excision, excised nodules removed	1·733	1·410
Mean differences, mean squares	0·05257	0·04415
A − B	—	−0·155 ± 0·068**
E − C	0·044 ± 0·111	0·125 ± 0·068
E − D	0·013 ± 0·115	—

ever, was not wholly confirmatory owing to the high counts obtained in the treatment to which nodules were added and on which no excisions were made. This result is completely at variance with the rest of the data and may be regarded as fortuitous. The apparent effect of addition and removal of nodules is here the opposite of what might have been expected, and does not lend any support to the supposition that the cut-off nodules may continue to have an inhibitory effect.

Further investigation of the relation between the number of excisions and degree of stimulation. In addition to expts. 2 and 3 two small experiments were set up to determine the effect of excision of up to twelve nodules. The first test was made on unselected material and the second with a commercial strain of red clover, the plants of which were first selected so that a large enough number was present on each plant to provide the maximum number of nodules it was proposed to excise. The results are set out in Table IX.

TABLE IX

Mean Log. Number (+1) of Infections and Dry Weight of Plants from which Varying Numbers of Nodules were excised

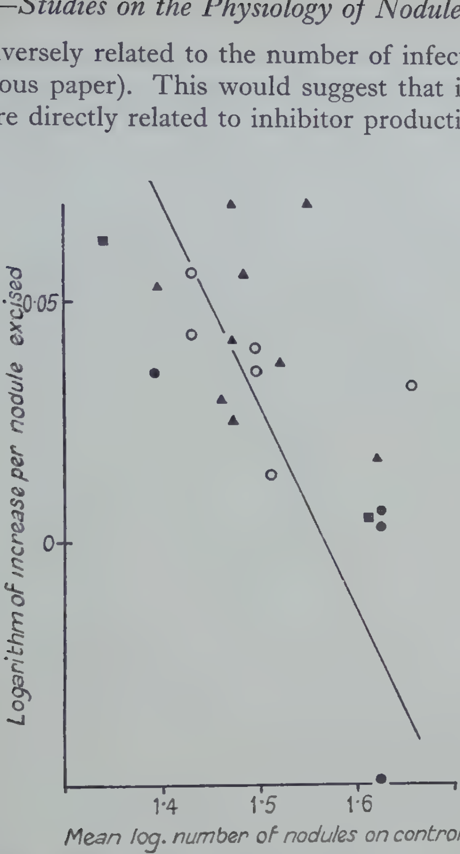
Number of nodules excised.	Mean log. number (+1) infections Experiment 3.	 Experiment 8.	Mean dry weight (mgm.) Experiment 3.
0	1·430±0·076	1·621±0·045	42·28±4·98
1	—	1·539±0·065	—
2	1·674±0·081	1·640 ,,	44·58±5·57
3	1·598±0·064	1·573 ,,	49·27±4·06
4	—	1·595 ,,	—
5	—	1·626 ,,	—
6	1·689±0·064	1·640 ,,	50·23±4·21
8	—	1·682 ,,	—
9	1·720±0·081	—	46·31±5·57
10	—	1·625 ,,	—
12	1·695±0·087	1·651 ,,	48·77±5·95

The replication was too small to obtain significant results for the individual treatments except in so far as expt. 3 is concerned, in which the results for removal of three and six nodules are significant (see Table III). The nodule number for the other treatments approach these significant values. Bearing in mind the uncertainty due to the small size of the samples the trend in the data may indicate an increasing effect as the number of nodules excised is increased. A similar trend is also seen in expt. 8, though here the general effect of excision is much less. It is not possible from the data to confirm this trend, though it is interesting to note that even where twelve nodules were excised at the start of the experiment, a number which is a high proportion of the nodules then on the plant, this has not prevented the formation of new nodules. Indeed, in these plants the rate of production of nodules was much higher than in the control plants. Any secondary effects, therefore, due to removal of a considerable amount of tissue and thus of potential nutrients to the plants, has not masked the stimulating effect of excision. Removal of nodules in this way deprives the plant of nitrogen for further growth, and therefore one would expect excision to have an effect for this reason on the final dry weight. The final weight of the plant will depend on the result of two opposing influences, viz. (i) the loss of potential nitrogen supply by the early removal of nodules, and (ii) the increasing nitrogen supply due to the simulation of new nodule production. It might be expected that the dry weight should show an optimum within the range of nodules removed. The final dry weights of plants for varying numbers of nodules excised in expt. 3 are shown in Table IX and indicate such an optimal relation.

Influence of source of plant material on degree of stimulation following excision. The increase in nodulation after excision evidently varies from experiment to experiment, the least stimulation occurring in expt. 8, in which each plant has been first selected to provide at least twelve accessible nodules for excision. This pre-selection clearly favoured abundantly nodulating plants and the very slight stimulation obtained suggests that the response is inversely related to the number of nodules produced. This is corroborated by the results of the companion experiment with the plants remaining after the above selection, and on which less than twelve nodules were present at excision (see expt. 5, Table VIII). In this experiment stimulation per nodule excised is high and the mean number of nodules formed is low. Experiments 6 and 7 (Table VII) form a similar pair of tests conducted respectively with sparsely and abundantly nodulating selections and show the same result.

This negative correlation between response to excision and susceptibility of the plant is illustrated in Text-fig. 7 for all experiments in which three-, four-, and six-nodule excision were made. It should, however, be pointed out that the estimate of the stimulation is dependent upon the estimate of the control values; if the estimate of the control is low, the amount of stimulation will appear high and vice versa. It is not appropriate, therefore, to determine the significance of this correlation from experiments in which the number of nodules has not been determined by selection. Accordingly the calculation of the regression in Text-fig. 7 is restricted to the two sets of experiments with selected plants, noted above, and represented in the figure as black circles and squares, and to expt. 2, which was conducted with nine separate strains of clover (black triangles). The calculated regression line ($b = -0{\cdot}2312 \pm 0{\cdot}0931$) will, however, embrace all the points represented, including those excluded from the calculation, which are shown as open circles.

The fact that some control plants produce a larger number of nodules indicates either that they produce less of the inhibitor or are less sensitive to its action. Removal, therefore, of a fixed number of nodules may be expected to have less effect on further nodulation on an abundantly nodulating plant, as indeed is shown to be the case. In this connexion it should be noted that the average nodule length and therefore the activity of the nodule meristem is known to be inversely related to the number of infections induced (see pp. 277, 278, previous paper). This would suggest that inherent differences in rooting habit are directly related to inhibitor production.



TEXT-FIG. 7. Relation between stimulation following excision and susceptibility of host plant: ▲ expt. 2, ● expts. 5 and 8, ■ expts. 6 and 7, ○ remaining experiments.

DISCUSSION

The above study has demonstrated that the development of a meristematic initial on the root is determined to a large degree by the activity of other meristems present, the results fully supporting the hypothesis that this mutual interaction is effected by a growth inhibitor produced locally in the meristems.

The stimulation of nodulation following excision recalls that found after delayed inoculation (Nutman, 1949 *a*). In the first the increase in rate of infection is temporary, a return to the rate characteristic for the individual plant taking place after some weeks, and in the second the curves for delayed and control sets run parallel. On the proposed hypothesis the following sequence of events may be presumed to take place. In the seedling, inhibitor production in the multiplying growing-points increasingly restricts development and leads to a progressive decline in the rate of growth. Excision causes a temporary reduction in the rate of inhibitor production and this is at once reflected in an increase in the rate of initiation of meristems compared with controls. After an interval the larger number of nodules now present on the root in their turn gradually influence the rate of appearance of new initials until the lower rate corresponding to the controls is regained. These changes are illustrated in Text-fig. 2, which refers to the total number of infections sustained by the plant in the sense that the numbers shown always include the number of nodules removed.

Consideration must now be given to the possible mode of action of the inhibitor as far as may be suggested by the above experiments. It is evident in the first place that the inhibition of the development of new meristematic areas, either lateral roots or nodules, is limited to the younger parts of the root in which such meristems can arise, whereas the nodules which are a source of the inhibitor are situated on the older parts of the root. The inhibitor thus acts at a distance; operations carried out on one part of the root induce changes elsewhere. The fact that the inhibitor acts on both lateral and nodule production is in accordance with the view already expressed that the nodule is a metamorphosed lateral root (Nutman, 1948).

The postulated inhibitor may reach the potential meristematic areas either by translocation within the plant or through the external medium into which it may be excreted by the meristems. Evidence for such a secretion has already been published (Nutman, 1945), and further work on this aspect will be presented in the next paper of this series.

It has been emphasized above that with effective strains the stimulation of nodulation following nodule excision is a little greater than that following root excision, but that the contrary obtains with respect to the stimulation of lateral roots. If the movement of the inhibitor is within the plant, these results suggest that the foci susceptible to infection in the root-hair zone are nearer to the sites of nodule excision than to the sites of root-tip excision and that the root foci are nearer to excisable roots than to excisable nodules. Such conditions would be satisfied by the following linear order of succession, starting from the root-tip: nodule foci, excisable nodule, root foci, excisable root. No data are available on the exact location of these structures, although this order agrees with the view that the foci which develop within the growing root-hair zone form nodules and those outside form laterals. The effect of changes in inhibitor production due to root-tip excision will also depend upon whether the stump of the excised root itself bears a susceptible zone and inhibited foci. In most cases in the above experiments root-tips were excised from laterals recently emerged from the primary root and without a root-hair zone. A very detailed study of the development of the root would be necessary for firm deductions to be made of the movement of inhibitor within the root.

If, on the other hand, the inhibitor is excreted from the meristem and is then taken up by the root, the root-hair zone which is known to be the seat of most active uptake of external material will be the region most affected and nodule formation may be expected to be most readily stimulated by excision as Text-figs. 3 and 6 show.

The inherent capacity of the plant to produce nodules or laterals has been shown to be a determining factor in the reaction to excision. Plants which normally produced few nodules are characterized by a large nodule size and such plants show a greater response than those which normally produce more numerous and smaller nodules. It is also known that plants of a given genetic constitution react to effective and ineffective strains by producing relatively few and large nodules in the first case and many small nodules in the second. The reaction to ineffective strains therefore is only the last term in a graded series of response to excision depending upon size and number of nodules produced. In Text-fig. 7 the negative correlation between nodule number in plants of varying genetic constitution and the magnitude of the stimulus to excision is shown to hold over a wide range of variation in nodule number. Accordingly attention must be centred on the inhibitory activity of the nodule itself. Experiments have shown that this activity is located in the terminal meristem of the nodule, since the removal of the meristem alone has as great effect as the removal of all the bacterial tissue. It has also been shown that an incision into the nodule, thus isolating the meristem, has an equally marked effect on inhibitor production. This would indicate first that the terminal meristem of the nodule and not the bacterial tissue is the source of the inhibitor, and second, that the transfer of some substance through the nodule tissue plays a part in the production of the inhibitor or in the transfer of the stimulus about the root. The known presence of an adventitious vascular system within the nodule would lend anatomical support for such a view, as also does the report of Harris et al. (1949) of rootlet development from the periphery of old nodules of *Sesbania grandiflora*.

The isolated nodule also appears to be inactive as an inhibitor as the experiments show in which such isolated nodules were added to the culture medium in which plants were grown. That the metabolic activity of isolated nodules rapidly ceases is indicated by the experiments of Burris et al. (1943) on nitrogen fixation by excised nodules, who showed that whereas an excised nodulated root system continues to fix nitrogen for a short time, fixation by excised nodules is so slight that its detection is in some doubt; presumably inhibitor production follows a similar course.

SUMMARY

The suggestion made in the earlier papers of this series that nodule formation is influenced by the production of an inhibitor in the growing-points of root and nodule has been examined in a study of the effect of excision of nodules and root-tips on subsequent nodule and root development. The excisions were carried out under sterile conditions with a micro-guillotine described in the text. The following results were obtained.

1. With effective strains of bacteria the excision of nodules or of root-tips causes a significant and transient increase in the rate of subsequent nodule formation, a slightly higher stimulation following nodule excision than

root-tip excision. Plants on which excisions have been carried out reach a higher final dry weight than untreated plants.

2. With effective inoculation the excision of the nodule meristem leaving the rest of the nodule attached to the plant, or the isolation of the nodule meristem by means of a transverse incision, is as stimulating to further nodule formation as complete excision.

3. No inhibition of nodule formation is obtained by adding cut-off nodules to a plant culture.

4. The amount of stimulation following excision of effective nodules is directly related to the number excised and inversely related to the inherent susceptibility of the plant; stimulation is low with abundantly nodulating strains of plant and high with sparsely nodulating strains.

5. Root-tip excision with effective inoculation is only slightly stimulating to further rooting.

6. With ineffective symbioses, either with ineffective strains of bacteria or hereditarily ineffective lines of plant, no stimulation of nodule formation follows nodule excision, though there is a slight response to root-tip excision approaching a level of statistical significance. Excised plants reach a higher final dry weight than untreated plants.

7. Plants inoculated with ineffective strains of bacteria from which nodules or root-tips have been excised produce larger numbers of lateral roots than do control plants.

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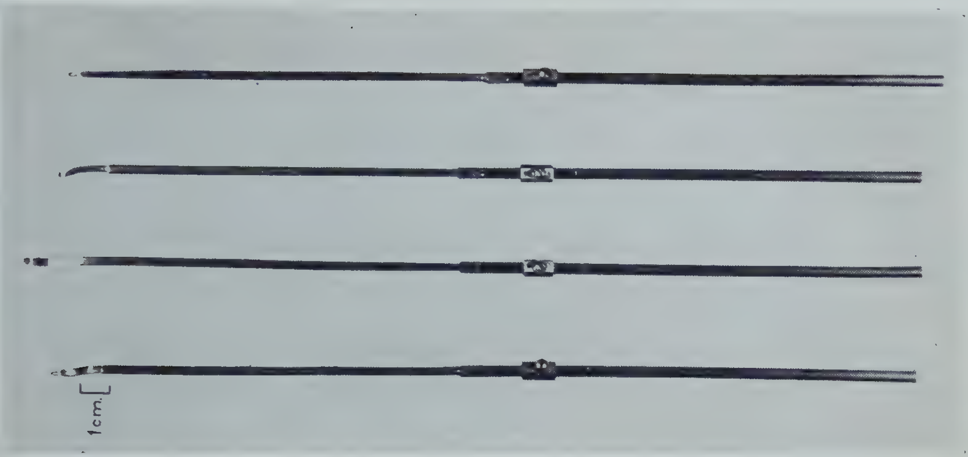
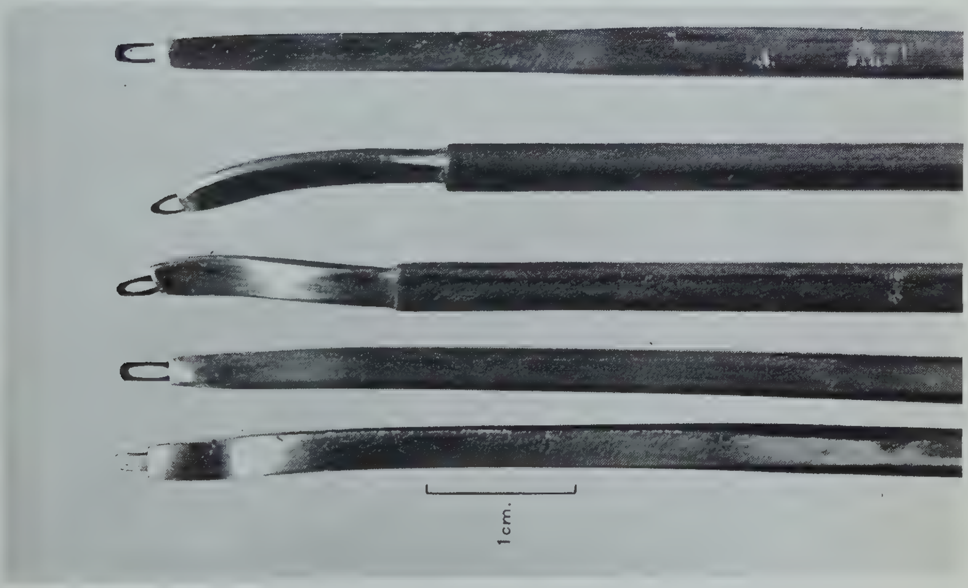
DESCRIPTION OF PLATE VII

Illustrating P. S. Nutman's article on 'Studies on the Physiology of Nodule Formation. III. Experiments on Excision of Root-tips and Nodules'.

Guillotine used for excision of nodules and root-tips.





P. S. NUTMAN

Studies in the Physiology of Parasitism

XVI. Effect of the Condition of Potato Tissue, as modified by Temperature and Water-content, upon Attack by Certain Organisms and their Pectinase Enzymes

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ABSTRACT

1. Certain bacteria which are normally termed saprophytic, viz. *Bacillus subtilis* and *B. megatherium*, are able to parasitize living potato tissue at a suitably high temperature or when the tissue is injected with water.

2. Within the group of four bacteria tested, there is a correlation between capacity to attack potato tissue and amount of pectinase enzyme excreted under standard conditions.

3. A qualitative difference between the pectinase enzymes of *Botrytis cinerea* and *Bacterium carotovorum* has been demonstrated. Preparations of the bacterial enzyme, which when tested on turgid potato discs of standard thickness were found to be weaker than *Botrytis* enzyme, were able to attack normal (subturgid) potato tissue, whereas the *Botrytis* enzyme failed to do so. No explanation of this difference is yet forthcoming. It does not seem to rest upon osmotic differences between the two enzymic preparations.

4. Rate of diffusion appears to be a limiting factor in the attack of potato tissue by preparations of pectinase enzyme.

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A. INTRODUCTION

IN the preceding number of this series (1937) Fernando showed that the nature of the nutrient medium had an important effect upon the secretion and properties of the pectinase enzyme secreted by a number of fungi and

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bacteria. Considerable importance attached to the pH drift in the culture solution, inasmuch as this affected the rate of growth of the micro-organism, the capacity of the latter to secrete pectinase, and to some extent the precise properties of the enzyme itself. A parallel investigation was carried out with potato tissue as substrate, the object being to determine how far the conditions in the latter, as modified in certain ways, influenced the vigour of parasitic attack by a range of micro-organisms. The following is an account of this work.[1]

B. Materials and Methods

The organisms used were *Bacterium carotovorum*, *Bacillus subtilis*, *Bac. megatherium*, *Bac. mycoides*, and *Botrytis cinerea*. Of these only the first and last are reckoned as plant pathogenic organisms. The isolates of *B. carotovorum*, *B. subtilis*, and *Bot. cinerea* were the same as those used in the paper already cited; the remaining two were taken from stock cultures maintained in the Plant Bacteriological laboratory.

The potatoes used were of the variety 'King Edward', apart from one experiment with 'Epicure' tubers. For inoculations of living tissue, tubers were first peeled, immersed in 0·1 per cent. mercuric chloride for 15 minutes, and rinsed for the same time in sterile distilled water. They were then transferred to sterile jars, cut with a flamed knife, and the inoculum spread over the cut surface.

To obtain enzymic preparations from the bacteria the pressed juice from rotted tissue was cleared by centrifuging and poured into three volumes of 95 per cent. alcohol. The precipitate was dried *in vacuo* over calcium chloride, ground, and when required was extracted at the rate of 0·25 g. of enzyme powder to 20 c.c. distilled water. For the preparation of *Botrytis* enzyme, Brown's (1915) technique was followed, the nutrient solution consisting of glucose, 2·5 per cent.; asparagin, 1 per cent.; KH_2PO_4, 0·5 per cent.; $MgSO_4$, 0·25 per cent.

Comparisons of enzymic activity were made by noting the time required (R.T., i.e. reaction time) for the maceration of discs of potato tissue one half-millimetre thick. These were cut on a microtome, and for any set of comparisons all were taken from the medullary tissue of the same tuber. For each determination three discs were used and the average R.T. quoted.

Estimations of pH values were made with the Universal Buffer Solution prepared by British Drug Houses according to the formula prescribed by Prideaux and Ward (1924). The usual indicators were used.

For quantitative work it was desirable to expose a definite area of host tissue to the action of a definite quantity of inoculum or of enzyme. For this purpose cylinders 3 cm. in diameter were cut with a cork-borer from the intra-vascular tissue of potato tubers. These were divided into lengths of about 2 cm. each, with parallel ends, and sterilized by 10 minutes' immersion in 0·1 per cent. $HgCl_2$ followed by two successive washings of 15 minutes each in sterile distilled water. They were then placed upright in glass capsules. One-half of a cubic centimetre of inoculum or of enzymic solution was then pipetted on each, there being no run-off in the circumstances. After a definite interval, the weight of rotted tissue was determined by subtraction.

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[1] This was carried out just before the outbreak of war and its publication held up until further extensions had been made. It is only in recent years that this type of investigation has again been taken up; hence the delay in publication.

The sterilization procedure just described has the effect of materially raising the water-content of the experimental tissues. It was often necessary to avoid this. In such cases the tubers, while still whole, were washed and surface-sterilized, and the experimental cylinders cut with the usual aseptic precautions only.

C. EXPERIMENTAL RESULTS

i. *Parasitism induced by rise of temperature*

The strain of *B. carotovorum* used strongly attacked potato tissue at ordinary temperatures, in contrast to the other three bacteria. At higher temperatures, however, these so-called saprophytes produced some attack, as shown in Table I. A number of control tubers equal to those inoculated were kept for the same time and at the same temperatures, and at the end of one week these showed no attack.

TABLE I

Effect of Temperature on Attack

Organism.	Temp. ° C.	No. of tubers. Inoculated.	Attacked.
B. subtilis . .	37	18	18
	30	10	0
	25	10	0
B. megatherium .	37	18	18
	30	10	10
	25	10	0
B. mycoides . .	37	18	0
	30	10	0
	25	10	0

B. subtilis and *B. megatherium* attacked all tubers at 37° and the latter those at 30° also. *B. mycoides* was ineffective at all temperatures. Though *B. megatherium* attacked over a wider temperature range than *B. subtilis*, its rate of attack was comparatively slow. The enhanced effect of higher temperatures on the attack of potato tubers by *B. subtilis* has already been noted by van Hall (1902).

The behaviour of the four bacterial species when grown on potato extract at 25° C. is shown in Table II with regard to pH drift and to the activity of exoenzyme produced.

TABLE II

pH Drift and Enzymic Activity in Cultures

Organism.	Initial pH.	2 days. pH.	2 days. R.T. (hrs.).	6 days. pH.	6 days. R.T. (hrs.).
B. carotovorum	5·9	6·5	4·75	7·9	< 0·5
B. subtilis	,,	7·1	3·75	8·4	1
B. megatherium	,,	5·2	> 48	8·0	6
B. mycoides	,,	5·2	> 48	7·0	> 24

All organisms finally brought about a pH drift towards alkalinity which was most pronounced with *B. subtilis* and least with *B. mycoides.* The last-named together with *B. megatherium* showed a preliminary drift to the acid side. It will be noted that the order of enzymic activity, as shown in the last column of the table, is substantially that of their capacity to attack potato tissue. At the top of the series is *B. carotovorum*, which is a normal parasite of potato; at the other extreme is *B. mycoides*, which does not attack potato even at high temperatures; intermediate are *B. subtilis* and *B. megatherium*, of which the former produces the more vigorous attack at 37°.

The parasitism of *B. subtilis* and *B. megatherium* at high temperatures (Table I) may be due in part to more vigorous growth or greater enzymic activity. Thus the optimum for growth of *B. megatherium* is about 35° C., that of *B. subtilis* between 37° C. and 40° C., and the optimum for the activity of the enzyme of *B. subtilis* was shown to lie above 37° C. There is evidence, however, that a greater susceptibility of potato tissue is produced by exposure to somewhat high temperatures. This was shown by the following experiment:

A batch of potatoes of the variety 'Epicure' was divided into two lots, one of which was kept at room temperature, the other at temperatures of 38° C. or 40° C. After a number of days the potatoes were peeled and sterilized in 0·1 per cent. mercuric chloride in the usual way. One half of each tuber was inoculated with *B. subtilis*, and the other half was kept as a control. Both inoculated and control halves were incubated at 26° C.

All the controls and all the inoculated half-tubers which had been stored throughout at laboratory temperature remained sound, whereas tubers which had been exposed to a high temperature (38° for 8 days or 40° for 3 days) before inoculations were all attacked. The tissue exposed at 38° or 40° for the times indicated was quite alive inasmuch as sample discs were plasmolysable in hypertonic sugar solutions, after which they became fully turgid when again placed in distilled water.

A similar increase in susceptibility, due to varying conditions of storage, was observed by Vasudeva (1930) with regard to the resistance of apple tissue to *Botrytis Allii.*

ii. *Parasitism induced by imbibition of water*

Whereas *B. subtilis* is unable to attack normal potato tissue at ordinary temperatures, it does so when the tissue is injected with water. Cylinders were cut from tubers and sterilized according to the method already described (p. 104). They were then injected with sterile distilled water under a filter-pump. For comparison, cylinders exposed for 4 hours to chloroform vapour, which was then removed by evacuation, and others which had been steamed for 30 minutes on three successive days were included in the experiment. All cylinders were then inoculated in a similar manner with *B. subtilis*. The results are given in Table III. With steamed tissue, which has already lost most of its coherence, the limits of attack could not be determined in the usual manner. Use was made of the fact that invasion of steamed tissue was marked by the development of a yellow colour.

TABLE III

Effect of Treatments of Potato Tissue on Attack by B. subtilis

	64 hours.			88 hours.		
		Extract of rotted tissue.			Extract of rotted tissue.	
Treatment.	Av. wt. of Rot (g.).	pH.	R.T. (hrs.)	Av. wt. of Rot (g.).	pH.	R.T. (hrs.).
Steamed .	1·80	7·9	2·5	3·6	8·0	2·5
Chloroformed	2·87	8·8	1	8·0	9·2	1
Injected .	0·32	6·7	4	1·5	7·4	2·25

The initial pH value of the potato tissue was about 6, and inoculation with *B. subtilis* caused an alkaline drift in all cases, especially in the chloroformed tissue, in which also the rate of invasion was greatest. There is an obvious correlation between the amount of rotting produced and the enzymic activity of the rotted tissue extract.

The experiment with steamed tissue merely proves that *B. subtilis* grows well on the ingredients of potato. The fact that maceration of chloroformed tissue took place means in addition that the organism is able to break down cell-walls which are probably not much different from those in untreated tissue. The results with injected tissue are the most interesting, as the cells, though not in a normal environment, are definitely still alive. Probably the only important changes consequent upon the injection arise from the replacement of air in the intercellular spaces by water and the fall of the suction-pressure of the host cells to zero. A similar effect of injection has been described by Clayton (1936) for the leaf-disease of tobacco caused by *Bacterium tabacum.*

The behaviour of *B. subtilis* on potato tissue, injected or uninjected, was compared side by side with that of its pectinase enzyme. The latter was prepared from fresh potato inoculated with the bacterium and incubated at 37° C. (p. 105). In the experiment of Table IV, five potatoes were used, from each of which four cylinders were cut, one for each of the four treatments under examination. The figures in the table give the weights of rotted tissue after 40 hours at 25° C.

TABLE IV

Comparison of Bacterial Inoculum and Enzyme of B. subtilis *on Normal and Injected Potato Tissue*

	Normal (water content 74%).		Injected (water content 81%).	
	Bacteria.	Enzyme.	Bacteria.	Enzyme.
1	0·0	0·0	4·8	1·1
2	,,	,,	1·0	0·85
3	,,	,,	1·2	0·6
4	0·25	,,	7·3	0·9
5	0·0	,,	5·9	0·6

The amount of rotting is significantly increased by injection of the tissues with water, at the 100:1 and 20:1 level for enzyme and bacterial inoculum respectively. The effect of injection upon amount of bacterial invasion varies considerably from one potato to another; hence the lower degree of significance of the result obtained in that case.

A similar conclusion applies to *B. carotovorum* and its enzyme. This organism, however, attacks potato tissue in its normal condition and enzymic preparations made from it do likewise. The efficiency of both, however, is increased by injection of the host tissue. Thus the average amount of bacterial attack after 7 days at 24° of 7 half-potatoes was 0·93 g. as against 14·1 g. for the injected halves. The significance of this difference lay between 50:1 and 100 : 1. In a similar experiment with 16 potatoes and with *B. carotovorum* enzyme, the average amount of rotting of the normal halves in 24 hours was 0·22 g., that of the injected 0·71 g., these figures giving a probability of more than 100:1.

The difference between the enzyme preparations of *B. carotovorum* and *B. subtilis*, viz. that the former attacks cylinders of normal potato tissue and the latter does not, is due to the greater concentration of the enzyme in the former. In tests with *B. subtilis* enzyme and with a preparation of *B. carotovorum* diluted so as to give the same reaction time on standard potato discs, no difference in behaviour could be established. So far as is known, therefore, there is no qualitative difference between the pectinase enzymes of these two organisms.

Botrytis cinerea in its relation to potato tissue is similar to *B. subtilis*. Spores of the fungus germinate when placed on the cut surface of fresh untreated potato tissue but rarely produce any measurable attack. When, however, the tissue is injected with water, definite attack follows. The behaviour of *B. cinerea* enzyme towards potato tissue, and particularly as compared with that of *B. carotovorum* enzyme, was examined in some detail.

iii. *Comparison of enzymes of* Bacterium carotovorum *and* Botrytis cinerea

It was pointed out in the preceding number of this series (1937) that the optimum pH for activity of *B. carotovorum* enzyme was about 8 as against an acid optimum (*c.* pH 6) for *Botrytis* enzyme. A further difference has been established, viz. that a preparation of *Botrytis* enzyme is less effective on normal potato tissue than is a preparation of the same, or even somewhat longer, reaction time from *B. carotovorum.* This is shown in Table V, which gives the summarized result of two experiments, each carried out on 15 tubers and with the precaution that comparisons were made between halves of the same tuber. The enzyme preparations were set up at approximately their optimum pH values, viz. 7·6 for *B. carotovorum* and 6·2 for *Bot. cinerea.*

TABLE V

Comparison of B. carotovorum *and* Bot. cinerea *Enzymes*

	B. carotovorum.		*Bot. cinerea.*	
Expt.	R.T. (hrs.).	Rotting of cylinder (g.).	R.T. (hrs.).	Rotting of cylinder (g.).
1	0·75	0·24	0·5	0·0
2	1·0	0·16	0·66	0·0

Their differences shown in both experiments were significant at more than the 100:1 level. It is thus seen that the weaker *B. carotovorum* enzyme, as tested on thin standard discs (which are fully imbibed with water), is the more effective preparation when tested on a mass of normal (subturgid) tissue.

The effect of injection of tissue on the activity of *B. carotovorum* and *Bot. cinerea* enzymes is illustrated by the following figures, which are the averages of eight measurements.

B. carotovorum	enzyme on	normal tissue.	Wt. of	rotted pt.	0.21 g.
,,	,,	injected ,,	,,	,,	0·80 ,,
Bot. cinerea	,,	normal ,,	,,	,,	0·0 ,,
,,	,,	injected ,,	,,	,,	1·15 ,,

All the differences shown are highly significant, at the 50:1 level at least. It follows therefore that there is a factor associated with the subturgid condition of tissue which antagonizes the action of pectinase enzyme and to which the enzymic preparation of *Bot. cinerea* is more sensitive than is that of *B. carotovorum.*

Comparisons of the type shown in Table V were carried out at various times of the year and with a range of enzymic extracts prepared from *B. carotovorum* and *Bot. cinerea.* The results of thirteen such experiments are summarized in Table VI. In the last two of these, crude enzymic extracts were used, in the others extracts which had been purified by one precipitation with alcohol.

For each experiment a large number of tissue cylinders were cut, and these were sorted at random into batches of 10, one such batch for each of the four treatments (normal *v.* injected, *B. carotovorum* enzyme *v. Bot. cinerea* enzyme). Portions of the cylinders were used for water-content determinations, before and after injection under the air-pump. The test discs for R.T.

determinations were all cut from the same tuber in each experiment, so that there is a strict comparison of the data along each horizontal line of Table VI.

TABLE VI

Comparison of B. carotovorum *and* Bot. cinerea *Enzymes*

	Water content of tissue.		Attack by *B. carotovorum* enzyme.			Attack by *Bot. cinerea* enzyme.		
Expt.	Normal.	Injected.	R.T. (min.)	Normal tissue.	Injected tissue.	R.T. (min.)	Normal tissue.	Injected tissue.
1	79·2	81·2	50	0·63±0·05	2·1±0·31	45	0·5±0·12	2·4±0·31
2	79·6	83·0	50	0·48±0·04	1·22±0·05	45	0·38±0·04	0·99±0·07
3	—	—	60	0·48±0·11	0·61±0·02	30	0·82±0·03	1·53±0·13
4	80·2	85·0	50	0·42±0·03	1·6±0·15	20	0·55±0·05	3·0±0·05
5	79	—	50	0·60±0·07	—	50	0	—
6	—	—	45	0·19±0·05	1·57±0·09	30	0·20±0·08	1·12±0·09
7	76	81	45	0·44±0·04	1·9±0·01	40	0·11±0·02	1·03±0·02
8	75	81	40	0·87±0·06	2·6±0·15	40	0·27±0·04	1·93±0·20
9	—	—	45	0·60±0·05	1·06±0·16	30	0	0·57±0·07
10	77	78	45	0·50±0·10	—	40	0	1·18±0·15
11	77	79	40	0·29±0·07	2·9±0·10	40	0	0·95±0·20
12	74	80·5	30	0·34±0·05	1·52±0·02	35	0	0·73±0·04
13	73	80	30	0·47±0·05	1·7±0·10	30	0·24±0·03	0·56±0·04
Av.	76·8	80·9	44	0·475	1·77	35	0·28	1·45

The enzymic preparations of *Bot. cinerea* were on the whole more active than those of *B. carotovorum*, as shown by the shorter reaction times on standard discs (overall average, 35 minutes as against 44 minutes). Nevertheless the average amount of attack on normal potato tissue was nearly twice as great by the latter, viz. 0·475 g. as compared with 0·28 g. Where enzymic preparations of about the same strength were used, as in expts. 5, 7, 8, 10–13, the amount of decomposition brought about by *B. carotovorum* enzyme was much the greater.

In the experiments in Table VI a certain amount of attack was obtained on normal potato tissue with *Botrytis* enzyme. The amount was greatest on tubers with highest natural water content (contrast expts. 1, 2, and 4 with 10–13). No doubt also the concentration of enzyme used also affected the result as in expts. 3 and 4.

Raising of the water content by an amount which averaged 4 per cent. markedly increased the amount of attack. The increase was 3- to 4-fold for *B. carotovorum* and 4- to 5-fold for *Bot. cinerea* enzyme.

Though most of the experiments of Table VI were carried out with somewhat purified enzymic preparations, the possibility still existed that impurities present, even after alcohol purification, were responsible for the difference in behaviour shown. A more strict comparison was set up by two methods, in the following manner.

In the first, crude enzyme preparations of *B. carotovorum* and *Bot. cinerea* were thrown down in 4 volumes of alcohol and the precipitates dried and ground with quartz sand. The powders were then extracted according to the scheme:

(*a*) *Botrytis* powder extracted in given volume of water,
(*b*) *B. carotovorum* extracted in the same volume of water,
(*c*) *Botrytis* extracted in the same volume of (*b*) which had been deactivated by boiling.

All three preparations gave the same reaction time (30 minutes) on standard discs, but only (*b*) attacked cylinders of fresh potato tissue. If the activity of (*b*) is conditioned by impurities associated with *B. carotovorum*, substantially the same impurities should be present in preparation (*c*). Yet the latter was ineffective.

For the second comparison preparations of *Botrytis* and *B. carotovorum* enzymes were diluted to half-strength according to the following scheme:

(*a*) *Bot. cinerea* enzyme+distilled water.
(*b*) *B. carotovorum* enzyme+distilled water.
(*c*) *Bot. cinerea* enzyme+deactivated *B. carotovorum* enzyme.
(*d*) *B. carotovorum* enzyme+deactivated *Bot. cinerea* enzyme.

All the preparations had a R.T. of 0·75–1 hour; when tested on normal potato cylinders, only preparations (*b*) and (*d*) were active. Preparations (*c*) and (*d*) must be very similar as regards their content of thermostable substances; nevertheless they reacted differently. A distinct qualitative difference between the pectinase enzymes systems of *Bot. cinerea* and *B. carotovorum* is thus indicated.

iv. *Factors which interfere with action of enzymes*

Throughout this work it was obvious that the amount of disintegration produced when a small amount of enzymic solution was laid on the surface of a cylinder of tissue was much less, even in the most favourable circumstances, than might be expected from the rate of destruction of test discs in the standard method of estimating activity. Thus a particular extract might decompose discs of 0·5 mm. thickness in half an hour, yet when the same extract was laid upon the surface of a large piece of tissue, the depth to which corrosion took place might be only 1–2 mm. in 24 hours. An interfering factor, depending perhaps on the quantitative relationship of amount of enzyme to amount of tissue in contact with it, seemed to be indicated. The following experiments were devised in further examination of this point.

Discs of different thickness (0·5, 1, 2, and 4 mm.) were cut and tested with an excess of the enzymic solution in the standard way. The tests were arranged so that the amount of tissue used was the same in all cases, i.e. 1 disc of 4 mm. thickness, 4 discs of 1 mm. thickness, &c. The results are set out in Table VII.

The reaction times increase approximately according to the square of the thickness of the discs; thus for the *B. carotovorum* pectinase acting on potato discs, the times for decomposition of discs of thickness 0·5, 1, 2, and 4 mm. are in the ratio of 1:5:14:46, which is not very far from the theoretical 1:4:16:64. Bearing in mind that the discs are exposed to enzyme on both

TABLE VII

Effect of Thickness of Tissue Discs on Time required for Maceration by Enzyme

	R.T. in hours.			
	Potato discs.		Carrot discs.	
Thickness of discs (×0·5 mm.)	Pectinase of *B. carotovorum.*	Pectinase of *Bot. cinerea.*	Pectinase of *B. carotovorum.*	Pectinase of *Bot. cinerea.*
1	0·66	0·4	4·75	3·5
2	3·5	2·9	18	13·5
4	8·75	7	41	> 48
8	31	23	> 48	> 48

sides whereas the cylinders are exposed only on one side, one can readily calculate that an extract which decomposes a disc 0·5 mm. thick in half an hour can be expected to corrode the surface of a cylinder to a depth of only 1·5–2 mm. in 24 hours, which is about the depth observed under favourable conditions.

The relation shown in Table VII between reaction time and thickness of disc is what would be expected if diffusion of the enzyme into the tissue obeyed Fick's Law. That a physical factor limits the speed of the reaction was further suggested by determining its temperature coefficient. For discs of standard thickness (0·5 mm.) and for a preparation of *B. carotovorum* enzyme of high activity the average Q_{10} of activity was determined as 1·39±0·11 over the range of 15–36° C. With greater thickness of discs the coefficient falls still lower, e.g. to 1·1 for discs of three times the standard thickness.

If one accepts that the rate of diffusion may be a limiting factor in the process of attack by the enzyme, it is readily understandable why injection of tissue, by replacing the air of the intercellular spaces with water, speeds up the reaction. But those considerations do not explain why non-injected potato tissue is usually not attacked at all by *Botrytis* enzyme. There must be some other factor to which *Botrytis* enzyme is particularly sensitive. When a limited volume of the latter is laid on the surface of normal potato tissue, the liquid sinks in in a short time and no disintegration of the tissue takes place. That the enzyme is present for some time after being absorbed can be shown by taking a surface section of the tissue and placing it in water. Sections so treated within about 8 hours from the time the enzymic solution was absorbed undergo maceration, thus proving that some enzyme is still present. After 24 hours it seems to have disappeared altogether.

When a small volume of *Botrytis* enzyme is placed on the surface of normal potato tissue, its effectiveness is definitely increased if at the same time an osmotic substance such as sucrose or glucose, which does not in itself retard the action of the enzyme, is also present. This is shown in Table VIII, the figures of which give the average amounts of rotting produced on potato blocks when 0·2 c.c. of *Botrytis* purified enzyme (R.T. 20 min.) diluted with

0·1 c.c. of the liquid indicated (water, sucrose, and glucose solutions) is placed on each block.

TABLE VIII

Effect of Osmotic Substances on Amount of Attack by Enzyme

Water.	Sucrose.		Glucose.	
	10%.	20%.	10%.	20%.
On fresh potato blocks 74·2% water.				
0·13±0·003	0·47±0·03	0·50±0·04	0·56±0·04	0·60±0·08
On injected blocks 77·6% water.				
1·38±0·14	1·52±0·11	1·93±0·12	1·76±0·16	1·94±0·14

The points brought out in the above table are as follows:

1. With fresh tissue the addition of either sugar to the enzyme gives a highly significant increase in the amount of rotting, the 10 per cent. solution being as effective as the 20 per cent. With injected tissue only the 20 per cent. concentration gives a significant increase.
2. Injecting the tissue with water is much more effective in increasing the rotting action than is the addition of either sugar to the enzyme.
3. There is an indication that glucose is more potent than sucrose in increasing enzymic activity on the solid blocks of tissue.

Thus while it is possible to increase the activity of *Botrytis* enzyme on blocks of potato tissue by preventing the osmotic withdrawal of water from the enzyme, the effectiveness of *B. carotovorum* enzyme, without such extraneous addition, still remains unexplained. The latter is either less sensitive to the loss of water brought about by the suction pressure of the cells or it prevents the abstraction of water from taking place by some immediate effect on the permeability of the potato cells.

D. DISCUSSION

The first main point of interest arises from the comparisons of four bacteria, viz. *B. carotovorum*, *B. subtilis*, *B. megatherium*, and *B. mycoides*. Though only one of these, viz. *B. carotovorum*, is pathogenic in the ordinary sense of the term, some of the others do attack under abnormal conditions, e.g. when the host tissue has been subjected to rather high temperatures or when it is injected with water. By this means one can arrange the four organisms in a descending order of parasitic vigour—viz. *B. carovotorum*, *B. subtilis*, *B. megatherium*, and *B. mycoides*. This is exactly the order in which they would be arranged on the basis of capacity to excrete pectinase enzyme under standard conditions. Such agreement can hardly be accidental, and so it appears that, within a limited range at least, there is a correlation between capacity to parasitize and capacity to excrete pectinase enzyme.

When, however, an organism with an entirely different type of metabolism, viz. *Botrytis cinerea*, is compared with *B. carotovorum*, the correlation between parasitism and excretion of enzyme, if it is present, is certainly masked. Thus it is possible to prepare from the fungus enzymic extracts which are fully as active as those of *B. carotovorum* when tested by a standard method, viz. by the immersion of discs of potato tissue in the extracts. Nevertheless, *B. carotovorum* attacks normal potato tissue whereas *Botrytis cinerea* usually does not.

If, on the other hand, small quantities of the enzymic preparations are laid upon normal potato tissue, the enzyme of *B. carotovorum* produces definite maceration whereas that of *Botrytis cinerea*, though it may be the more active when tested by the standard method, tends to be imbibed by the tissue and little if any demonstrable action results. Potato tissue which has been injected with water is attacked by both enzymes, and it is interesting to note that on injected potato tissue *Botrytis cinerea* is an active parasite. The close similarity in the behaviour of organism and enzyme gives a strong indication that the enzyme is in reality the active agent of parasitism.

A further result arises from the foregoing. There is in normal potato tissue a factor which counteracts the activity of pectinase enzyme. Its action is shown on the pectinase both of *Botrytis cinerea* and of *B. carotovorum*, but more severely upon the former, so that while the change from the injected to the normal condition of potato tissue slows down the action of the latter it usually completely inhibits the action of the former.

Further work will be required for the elucidation of this important factor. On the basis of results so far obtained, it does not appear to be of osmotic nature. Investigation might be directed to the question of possible interference by other enzymes—more particularly oxidizing enzymes—with the activity of pectinase.

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The Shoot Apex of Some Monocotyledons

I. Structure and Development

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With Plate VIII and six Figures in the Text

ABSTRACT

The form and structure of the apex of five genera of monocotyledons have been investigated according to methods which have already been applied to other groups. It is found that the growing-point exhibits a cyto-histological zonation: the meristem is organized into four distinct regions, the cells of which differ in appearance and properties and in their direction of division.

It is therefore suggested that the *tunica-corpus* concept is a convenient basis for the description of the apex in this group of plants. Both layers are derived from a small group of apical initial cells which in each species are clearly differentiated from the more active meristematic tissue to which they give rise. The corpus may be further subdivided into a central region of rib meristem where divisions are predominantly transverse, and a peripheral flank meristem which is actively concerned in leaf initiation.

The relationship of the growing-point and youngest leaf primordium is described and problems relating to leaf initiation are briefly discussed.

INTRODUCTION

THE structure and mode of growth of the shoot apex has periodically occupied the attention of plant morphologists, and during the past 20 years there has been a widespread revival of interest in these problems, as a result of the formulation of the *tunica-corpus* concept. The excellent reviews of Foster (1939, 1941) and Philipson (1947, 1949) have followed the history of this work in some detail.

The attempt to describe the shoot apex has passed through three phases. First came the apical cell theory of Nageli which we now know to be inadequate for the more complex plants. It was accordingly replaced by Hanstein's primary histogen theory which states that the meristem of the growing-point consists of three layers, the dermatogen, periblem, and plerome, each arising from a separate set of initials.

While the existence of stratification of the apex is an accepted fact, Hanstein also ascribed certain morphological implications to his layers. The activity of these histogens as specific issue-builders has never been demonstrated. In many apices, indeed, it is impossible to distinguish between periblem and

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plerome, so that rigid use of the descriptive terms lacks both reality and utility. In contrast, the *tunica-corpus* theory of Schmidt is more flexible in detailed interpretation. It envisages a central meristematic core of cells, the corpus, which is dividing in all directions and increasing the volume of the apex. This is surrounded by the tunica, an outer envelope of one or several layers of smaller cells dividing anticlinally and hence growing mainly in surface area. This is a more plastic conception which allows for fluctuation in the number of tunica layers. It should be emphasized that the delimitation of even these two simple zones is sometimes difficult.

In addition to this stratification, a cyto-histological zonation has been recognized in a number of plants by Foster et al. The value of these two conceptions in the description of the shoot apices of gymnosperms and dicotyledons has already been demonstrated, and it will therefore be useful to examine the shoot apices of some monocotyledons not previously described in the literature, with special reference to these ideas.

MATERIALS AND METHODS

This examination is based on species representing five genera: *Elodea densa*, *Convallaria majalis*, *Carex hordeistichos*, *Luzula sylvatica*, and *Narcissus pseudo-narcissus*. The species described represent a considerable range of variation in the shape of the apex, from the cylindrical form of growing-point in *Elodea* to the broadly conical types of *Carex* and *Narcissus*. They were also chosen for their ease of dissection and availability.

Shoot tips were collected for investigation at different times of the year, but the observations are largely based on material fixed during the season of most active growth. In all, between 30 and 60 apices of each species were examined.

Dissection. The growing-point is exposed by removing the leaf primordia with a fine mounted needle, a binocular dissecting microscope being used for the later stages of dissection (Sharman, 1943). Speed is important, since the growing-point shrivels rapidly on exposure to air. Apices were examined and drawn under the binocular and others were mounted and stained in the solid (Matske, 1948; Sharman, 1945).

Sectioning. Dissected apices were fixed in chrome acetic acid and embedded in paraffin wax, and serial sections cut at 10 μ on a rotary microtome. Sections were stained according to the iron-haematoxylin technique of Heidenhain.

ZONATION OF THE APEX

The following account of the form and structure of the shoot apex is based on the examination of dissected apices and longitudinal and transverse serial sections of the growing-point, to give a solid interpretation. It is found that the growing-point consists of a number of distinct zones which will be described separately, although it should be remembered that the individual units are organized and act together as a whole.

Tunica

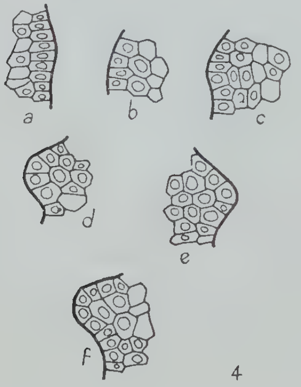
The superficial tissue of the growing-point consists of rectangular cells dividing regularly and anticlinally to form a distinct layer or layers of cells, which, provided that the fluctuation in depth is accepted as part of the definition, can best be described as a tunica. Text-figs. 1, 2, and 3 and Pl. VIII, A and B, show that in *Elodea*, *Convallaria*, and *Carex* the tunica is constantly uniseriate, but *Luzula* (Pl. VIII, D) shows some variation in the number of layers present, though there are frequently two or three. In *Narcissus* (Pl. VIII, C) it was often found difficult to determine the exact depth of this zone, especially down the sides of the apex, but it is fairly certain that it may sometimes be as much as five cells deep but more commonly three or four. It is interesting in this connexion to note that the number of tunica layers varied in the three other species of *Carex* examined, though it was constant for the particular species, and similar variations have been reported for several other genera. Reeve (1948) describes fluctuations in the number of tunica layers in *Salix* and *Carrya* and similar observations have been made on *Viburnum* by Cross (1937). Although the constancy in direction of cell division makes the tunica a discrete unit or sheet of cells, this uniformity of appearance is destroyed by a differentiation within the layer. Round the sides of the growing-point where the tunica passes over the youngest developing leaf-primordium, the cells are more densely stained and often orientated with their long axes at right angles to the tangential surface of the apex, so that new walls are formed in a direction parallel to the long axis of the cell and not in the plane of minimal area. The cells of this region appear to be in a highly active state of division tightly pressed one against the other. In contrast to this, the tunica cells round the tip of the apex are frequently orientated parallel to the surface and stain more lightly. The cells appear to divide relatively infrequently, and it is often possible to distinguish three or four larger cells in the centre. These are the initial cells from which the tunica is derived (Pl. VIII, A and B).

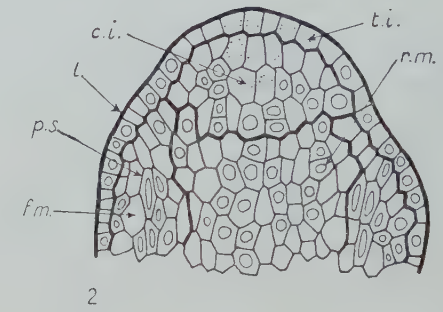
In none of the species examined has there been any evidence of the occurrence of periclinal divisions in the tunica, either round the tip of the apex or in the lateral regions of leaf initiation. This is contrary to some of the reports of other workers. In particular, Sharman (1940, 1942) observed periclinal divisions in the dermatogen of *Zea mays* and Rosler (1928) and Kleim (1937) both report that leaf primordia are initiated by periclinal divisions in the tunica of *Avena* and *Triticum*.

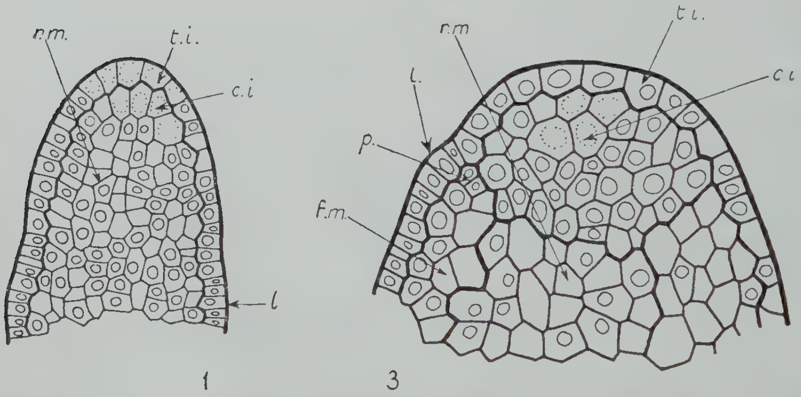
Corpus

Within the tunica mantle of cells is enclosed a central core of meristem, the corpus. This region of densely stained actively dividing cells is initiated by a small group of apical cells which are contiguous with and similar in appearance and properties to the tunica initials. The corpus is composed of two distinct zones.

Rib meristem. The central region of the corpus is characterized by the formation of new cell-walls predominantly in one plane, at right angles to the long axis of the shoot. Successive transverse divisions lead to the production of file or rib meristem, 'Rippenmeristem' of Schuepp. These vacuolated dividing cells are, according to Schuepp's definition, typically rectangular in shape and arranged with their long axes in the plane of cell division. In this work the term 'rib meristem' is applied to that central region of the corpus in which new walls are commonly transverse. Cells may occasionally divide longitudinally and there may be more irregularity in the orientation and arrangement of cells than is suggested by the rigid definition of Schuepp. In *Elodea* the organization is extremely regular and the cells are orientated transversely, each short file referable to the original parent cell (Text-fig. 1 and Pl. VIII, A). The other species show more variation. In *Convallaria* the cells are often arranged with their long axes parallel to that of the shoot (Text-fig. 2), or they may be isodiametric and can rarely be seen in the short cell files of constant number.







TEXT-FIGS. 1–3. L.S. growing-point. Thicker cell-walls denote boundaries of the different zones. 1. *Elodea densa* × 195. 2. *Convallaria majalis* × 195. 3. *Carex hordeistichos* × 550.

c.i., corpus initial; *f.m.*, flank meristem; *l.*, leaf primordium; *p.*, periclinal division; *p.s.*, pro-cambial strand; *r.m.*, rib meristem; *t.i.*, tunica initial.

TEXT-FIG. 4. *Elodea densa*: Stages in leaf initiation as seen in L.S. × 195.

Luzula shows characteristic files of rib meristem which arise from the corpus initial cells by a series of divisions which are cambial-like in appearance (Pl. VIII, D). A similar case has been mentioned by Ball (1941), who described cambial-like divisions by means of which the corpus initials added to the rib meristem in *Phoenix*. But there are no other references to this in the literature, and it has not been observed in any of the other apices examined during this work.

Longitudinal sections of the elongated apex of *Elodea* show that the rib meristem is very conspicuous in this plant. It extends for a considerable distance behind the corpus initials and gradually passes into a region where occasional longitudinal divisions occur. These become more frequent as distance from the apex increases and eventually predominate in the region where increase in the diameter of the axis is taking place. But the small convex apex of *Carex* shows very little longitudinal extent of rib meristem (Text-fig. 3) and it is difficult to distinguish the boundaries of the region in this species, since many of the cells are elongated in the plane of the long axis.

Flank meristem. Surrounding the rib meristem and derived laterally from the group of corpus initials is a cylinder of peripheral or flank meristem. In all the species examined this is characterized by increased density of staining, suggesting that the cells of this region are in a highly active state of cell division. The cells are smaller and show less evidence of vacuolation than the neighbouring partly vacuolated dividing cells of the rib meristem. In this respect they have more affinity with the lateral tunica.

This peripheral meristem corresponds in position with the increase in diameter of the growing-point associated with the youngest leaf primordium, which arises as a lateral protuberance. The rib meristem zone shows little increase in width after its inception.

The flank meristem extends some depth below the tunica in *Convallaria*, where it appears in longitudinal section as a fan-shaped zone of cells with large densely stained nuclei which appear to be dividing frequently (Pl. VIII, B). The majority of divisions are anticlinal and seem to correspond in frequency with the divisions in the lateral tunica which envelops this zone. For this reason it may be difficult to differentiate between the tunica and outer layers of the flank meristem, particularly in *Narcissus* and *Luzula* where there is more than one tunica layer.

There may also be a number of periclinal divisions in the tissue immediately beneath the tunica and particularly in the later stages of the initiation of a primordium (Text-fig. 3 and Pl. VIII, B). These divisions increase the bulk of the primordium, and also by increasing the depth of the flank meristem itself, push the developing leaf initial farther out, while the anticlinal divisions increase its surface area. Later some of the inner cells of the flank meristem elongate, becoming narrow with densely staining oval nuclei (Text-fig. 2). These cells constitute the procambial strand which is initiated during the first plastochrone.

In *Narcissus* the flank meristem is more extensive than in the other species and the rib meristem occupies a central zone of relatively small diameter (Pl. VIII, C). The opposite occurs in *Elodea* (Pl. VIII, A), where the corpus is composed almost entirely of rib meristem directly surrounded by the uniseriate tunica. The flank meristem consists only of two or three hypodermal cells which divide periclinally in the early stages of the initiation of a leaf. This striking difference in the organization of the corpus in the two species will be considered in more detail in a later paper.

The zone of initial cells

It has been indicated that both tunica and corpus are derived from separate groups of initial cells which are contiguous with one another at the tip of the apex and have certain features in common. These cells can usually be identified by their larger size and the appearance of their more vacuolated contents, which stain relatively lightly. The two initial zones differ in one respect. The tunica initials always divide anticlinally, replenishing the superficial tissue, which is being continually incorporated in the developing leaf primordia towards the base of the growing-point. Divisions in the corpus initial zone are more irregular and may take place in any plane, those produced laterally adding to the flank meristem, while the rib meristem is being restored in the centre.

The number of cells involved in this initial zone, and their detailed structure, varies in the different species. The rib meristem of *Elodea* is initiated by a group of four or five irregularly dividing cells with a high nuclear/cytoplasmic ratio (Text-fig. 1 and Pl. VIII, A). These initial cells have thinner walls which do not stain so readily as the other walls of the growing-point, and their large nuclei also stain more lightly. Their whole structure is less clearly defined than the other meristematic cells. Three or four large isodiametric cells of similar appearance can also be seen at the tip of the tunica, contrasting sharply with the small rectangular tunica cells down the sides of the growing point.

Convallaria also shows this apical differentiation. There is a group of large lightly stained cells, in which it may be difficult to distinguish the nuclear membrane, at the tip of both tunica and corpus (Text-fig. 2 and Pl. VIII, B). In longitudinal section, the tunica initials are more rectangular in shape and orientated parallel to the surface of the growing-point so that new walls are being formed in the plane of minimum area. The tunica gives the appearance of being stretched tangentially across the top of the apex, whereas at the sides the tunica cells are arranged with their long axes at right angles to the surface.

Similar differences in orientation of tunica initials and lateral tunica cells have also been observed in *Luzula* (Pl. VIII, D), where there is little differentiation in size. This species also shows a corpus initial zone of considerable extent, consisting of conspicuously vacuolated cells which are slightly larger than the adjacent meristematic cells and are clearly distinguished by irregularity in their planes of division and their polygonal shape in longitudinal section.

Carex shows a fairly large group of vacuolated initial cells occupying the top of the apical dome (Text-fig. 3). These are lightly stained and divide irregularly in all planes. Often two, three, or four of these cells appear considerably larger than the rest and more vacuolated. The walls of this region show a lower affinity for stains.

The massive growing-point of *Narcissus* exhibits little evidence of the differential staining observed in the other species (Pl. VIII, c). The tunica layers sometimes show two or three slightly larger cells at the tip, and in some apices the cells were orientated tangentially. A group of cells dividing irregularly and in all directions can be seen between the tunica and the tip of the rib meristem, but they show no significant difference in size or density of staining, although they undoubtedly constitute the meristem which initiates the corpus. Their only distinction lies in the direction of cell division, resulting in polygonal shaped cells.

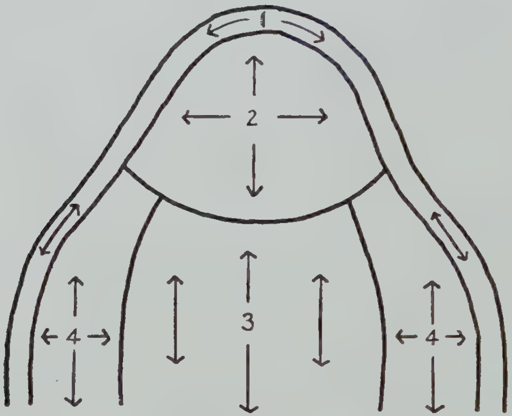
Every species examined shows the presence of a group of apical initials at the tip of the corpus and tunica layers, and the appearance of these cells suggests that they divide relatively infrequently compared with the actively dividing tissue of the flank and rib meristems. The characteristics of these cells are that they may be larger, more vacuolated, less densely stained, and with a higher nuclear/cytoplasmic ratio and thinner walls. There is a marked differentiation in appearance and content of the cells which in no way supersedes the zonation according to the disposition of the cell walls. The two are complementary features of apical organization.

This type of zonation was first recognized in the gymnosperms by Foster, who refers to the corpus initials as the 'central mother cell' zone on account of their role in adding cells to the active meristem behind. It has since been reported in many gymnosperms, in Ginkgo and the Cycads, and its occurrence has been recognized in angiosperms (Foster, 1941). Subsequently this zonation in the density of cytoplasm has been reported in *Phoenix* by Ball (1941), in *Sinocalamus* by Hsu (1944), and in various dicotyledons by Majumdar (1942) and Philipson (1947). It would seem to be a constant feature of all shoot apices.

The behaviour, appearance, and staining properties of this zone suggests that there is a difference in metabolism between these cells and the more meristematic ones which surround them. Attention was drawn to the frequent presence in higher plants of a group of larger cells at the tip of the growing-point by Priestley (1928), who suggested that they were concerned with protoplasmic synthesis, whereas the meristematic cells behind the apex are more easily supplied with nutrient and divide more rapidly. The latter may be more concerned with carbohydrate metabolism since they form new cell-walls more frequently and thicker ones. Priestley, however, stated that these cells were not present in angiosperms, except in *Elodea*.

When living apices of *Elodea* were stained in methylene blue the zone of initial cells stained immediately and more densely than the rest of the growing-point. Thus the differentiation is present in living tissue and not an artefact of fixation. This might suggest a difference in permeability in the initial zone. Staining of both live material and prepared sections indicates a difference in the nature of the cell-walls. It has already been noted that they are thinner than those in the rest of the corpus meristem, which supports Priestley's theory.

Sometimes the corpus initials do not show evidence of cellular enlargement or differential staining, but there are always present a number of cells dividing in all planes, a block meristem, or the 'massiges meristeme' of Schuepp. The cells which result from these divisions give rise to the corpus meristem.



TEXT-FIG. 5. Zonation diagram of hypothetical shoot apex showing direction of cell divisions. 1, tunica and initials; 2, corpus initial; 3, rib meristem; 4, flank meristem.

The shoot apex may then be summarized as consisting of four zones (Text-fig. 5):

1. The *tunica* and its initials, which divide anticlinally.
2. The *corpus initials.* A meristematic mass of cells from which the whole of the corpus is derived. A block meristem with divisions in all planes.
3. The *rib meristem* of the corpus which is concerned in elongation growth since all divisions are transverse.
4. The *flank meristem* which promotes the development of the leaf primordia by means of anticlinal and periclinal divisions.

The last three zones are all part of the corpus.

This dynamic interpretation of the organization of the shoot apex is a more convenient basis for description than the primary histogen theory of Hanstein. Sharman, however, retains a preference for the latter terminology in his description of various grass apices. The tunica with its conception of plasticity in depth of the superficial anticlinally dividing layers is not accurately equivalent to the dermatogen, which is defined as a single layer of cells. Sharman has therefore found it necessary to introduce the terms 'hypodermis' and 'sub-hypodermis' for the layers immediately beneath the tunica.

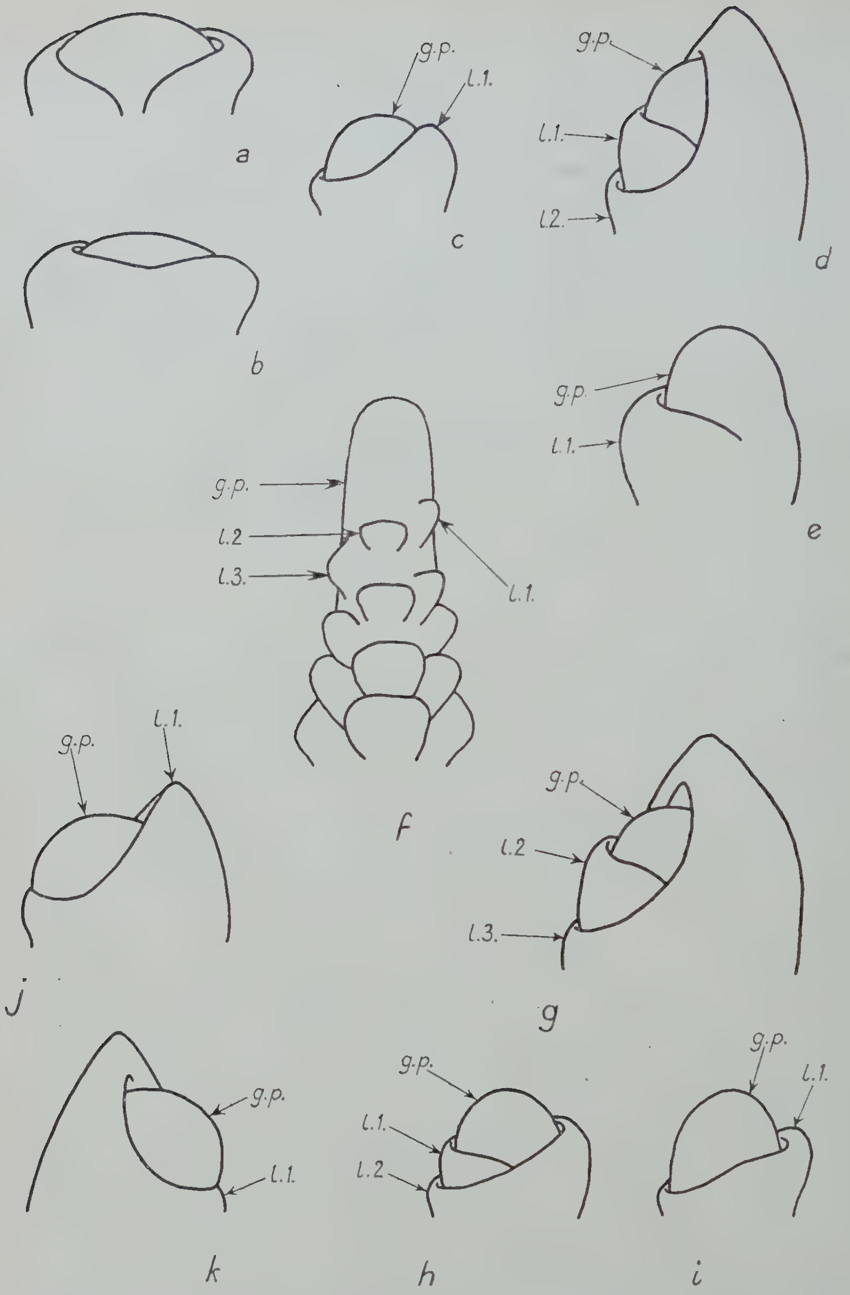
APICAL CELLS

The number of apical initials which showed size increase varied in the different genera and even within a species, but it was never found to be less than three and usually considerably more extensive. Thus there is no evidence of a single apical initial cell as described by Kleim (1937) and Rosler (1928) for *Avena* and *Triticum*. It was, however, observed in *Elodea*, where there were commonly four or five initials, that the number was often reduced to one or two in younger axillary apices. It seems possible that the initials may increase in number with the age of the apex, originating from a single one in the seedling. In describing the embryogeny of *Phlox drummondii*, Miller and Wetmore (1945) state that, as far as can be observed, a single apical cell gives rise to the initial cells of both dermatogen and corpus.

LEAF INITIATION

In all the apices except *Elodea*, where the leaves are formed in prolific numbers, it was impossible to trace in detail the stages in initiation and development of the leaf primordia. Text-fig. 6 gives diagrammatic representations of dissected apices showing growing-points at different stages and angles during the removal of the youngest leaf primordia. They give some idea of the shape of the growing-points and the extent to which they are enclosed by the first and subsequent leaves. The most essential histological feature present in each case was the activity of the tunica, the cells of which appear to be dividing rapidly in the anticlinal direction. In *Elodea* (Text-figs. 1 and 4*a*) this was always the first visible sign of initiation and preceded the periclinal divisions in the outer corpus layer beneath.

In the region of leaf formation the tunica cells are tightly packed together and the cell diameter appears particularly small at the point where a leaf is about to be initiated. This could be caused by a lateral pressure, which would presumably be greater in the region of most active cell division. The next stage of the primordium is seen as a bulge in the tunica as the cells expand a little, and at the same time the corpus cell immediately behind the superficial protuberance also vacuolates and enlarges (Text-fig. 4*b*). The tangential pressure is released allowing the cells of the future primordium to vacuolate, or, alternatively, the expansion of these cells itself releases the lateral pressure. The corpus cell concerned then divides periclinally (Text-fig. 4*c*), enlarging the bulge which is now the young leaf primordium. The cell at the tip of the primordium often expands more than the other tunica cells and it may become almost conical in longitudinal section. This isolated division in the corpus layer, the hypodermis of Sharman (1945), is followed by longitudinal divisions in the corpus cells immediately above and below (Text-fig. 4, *d* and *e*), so that altogether three corpus cells take part in leaf initiation. This is the only



Text-fig. 6. *a*, *b*, and *c*. *Narcissus pseudo-narcissus* (× 45). Three different views of the same apex. Growing-point (*g.p.*) and first leaf primordium (*l.*1.). *d* and *e*. *Convallaria majalis* (× 105). Two stages in dissection: *d*. growing-point and two youngest leaf primordia (*l.*1. and *l.*2.); *e*. growing-point and first leaf primordium. *f*. *Elodea densa* (× 95). Growing-point and young leaf primordia. *g*, *h*, and *i*. *Luzula sylvatica* (× 120). Three stages in dissection: *g*. growing-point and three youngest primordia; *h*. growing-point and two youngest primordia. *i*. growing-point and first leaf primordium. *j* and *k*. *Carex hordeistichos* (× 250). Two different views of the same apex. Growing-point (*g.p.*) and first leaf primordium (*l.*1.).

indication of flank meristem tissue in *Elodea*. The outer daughter cells of these three divisions continue dividing to form the limited amount of mesophyll which is present in the midrib region of the mature leaf. The tunica cells of the young primordium remain meristematic and continue dividing anticlinally to form the upper and lower epidermis (Text-fig. 4 *f*).

The other species have the sheathing leaf insertion typical of most monocotyledons, in which the leaf primordium is initiated on one side of the apex (Text-fig. 6). As development proceeds the cell divisions spread round the sides of the apex to complete the insertion, but the original growth centre maintains its initial ascendency and a hood-shaped primordium develops arching over the growing-point from the side of initiation. The extent to which this develops before a younger primordium arises varies in the different apices according to the length of the first plastochrone and the individual growth rate. This controls the number of primordia which may be seen immediately enfolding the growing-point when the apex is exposed, only one in *Carex* and *Narcissus* (Text-fig. 6 *j*, *k*, and *a–c*) but two in *Convallaria* (Text-fig. 6 *d*) and three in *Luzula* (Text-fig. 6 *g* and *h*). There are also differences in the rate at which the cell divisions spread round the circumference of the growing point.

There is a considerable lag in *Narcissus*, so that in longitudinal section older primordia are represented by small basal upgrowths on the surface of the stem axis and on the side opposite to their initiation (Pl. VIII, C). In *Convallaria* the initiation spreads rapidly to give a ring-like protuberance which is only slightly more developed on one side than the other (Text-fig. 6 *e*). In the mature leaf, the blade develops from the first formed part of the primordium, and the basal sheath represents the encircling spread of activity. In longitudinal sections it can be seen that this activity is confined to the tunica and flank meristem which is extensive in *Luzula*, *Carex*, and *Narcissus*. The cell-walls, cytoplasm, and nuclei of the lateral zones stain very densely, and the tunica cells appear tightly pressed one against the other as a result of the continuous and frequent anticlinal divisions which are always the first visible indication of leaf initiation. These are immediately followed by divisions in the flank meristem giving rise to the tissue from which the inner part of the leaf is derived. Anticlinal divisions predominate, accommodating the increasing surface area of the tunica, and periclinal divisions increase the bulk of the leaf initial, replacing the flank meristem which is used up, and pushing the young primordium out laterally on a buttress of flank meristem. Thus in the species with more massive leaves a considerable part of the corpus participates in leaf formation.

The idea that the superficial layers of the shoot apex must be in a state of tangential pressure is one of the essentials of Schuepp's folding theory of leaf initiation. The appearance and orientation of the tunica cells in the region of leaf initiation in those species examined support this suggestion. Frequently the diameter of these cells was so small that the two radial walls appeared to be pressed against the nuclear membrane, and in some cases the nuclei were oval in shape, which could presumably be caused by the pressure of adjacent cells on the side walls of the plastic meristematic tissue. Such a tangential pressure would inevitably result from a highly active state of cell division which is localized in one plane and not completely accommodated by the tissue within. The more vacuolate dividing cells of the rib meristem appear to have a lower rate of mitosis. This difference is particularly striking in *Elodea* (Pl. VIII, A), where the cells of both rib meristem and tunica zones are similarly orientated as seen in longitudinal section, but it should be investigated quantitatively before any definite conclusions can be drawn.

Schuepp suggested that lateral pressure in the outer layers of the apex was due to growth in surface area resulting from the predominance of anticlinal divisions which were not fully compensated by the volume growth of the meristematic core, in which cell divisions occur in all planes. This is only true for the initial zone at the tip of the apex, where cell divisions are relatively infrequent and the tunica appears to be in state of tension. The zone of leaf initiation lies farther back in the region where the inner corpus cells are dividing transversely, i.e. in the same plane as the tunica, and in one plane only. It is necessary to determine whether in fact the tunica is in a state of tangential compression or tension, and whether the condition found is uniform over the whole surface of the apex. Appearance suggests that the superficial cells round the tip of the apex and those at the sides may be subjected to stresses of a different nature.

The question of leaf initiation has been approached from other angles, particularly in relation to problems of phyllotaxis. Priestley and Scott (1933) put forward the repulsion theory which embodies the physiological conception of competing growth centres at the apex, leaf primordia arising as far as possible from previously formed initials. It has also been suggested that phyllotaxis and vascular development are interrelated, and Sterling (1945) favours this view, pointing out that leaf traces are formed before the leaves in *Sequoia sempervirens*. Both these conceptions may be subjected to physiological interpretation involving growth-promoting substances or nutrient supply, but more recently Snow and Snow (1947) have resolved the question into one of spatial relationships. Their results indicate that a new leaf is formed in the first available space, its position depending on the way in which existing leaf bases occupy the surface of the apex.

This purely physical or geometrical theory and previous physiological explanations are not comparable with the one proposed by Schuepp which explains how initiation takes place. There are two separate problems.

CONCLUSIONS

We should now have a three-dimensional picture of the continuous development of the growing-point. The meristematic layers are derived from groups of large initial cells which divide infrequently. Leaves are being formed on the sides of the apex by the activity of the flank meristem and tunica of this region. As the superficial meristem is incorporated in the leaf primordia, the flank meristem within increases the diameter of the apex. At the same time the developing leaves are pushed farther back by the elongating activity of the rib meristem which is responsible for the growth in length of the apex. The activity of these two zones and the balance between them is an important factor in the form and organization of the growing-point, and in the whole plant, since structure is determined in this region.

ACKNOWLEDGEMENTS

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DESCRIPTION OF PLATE

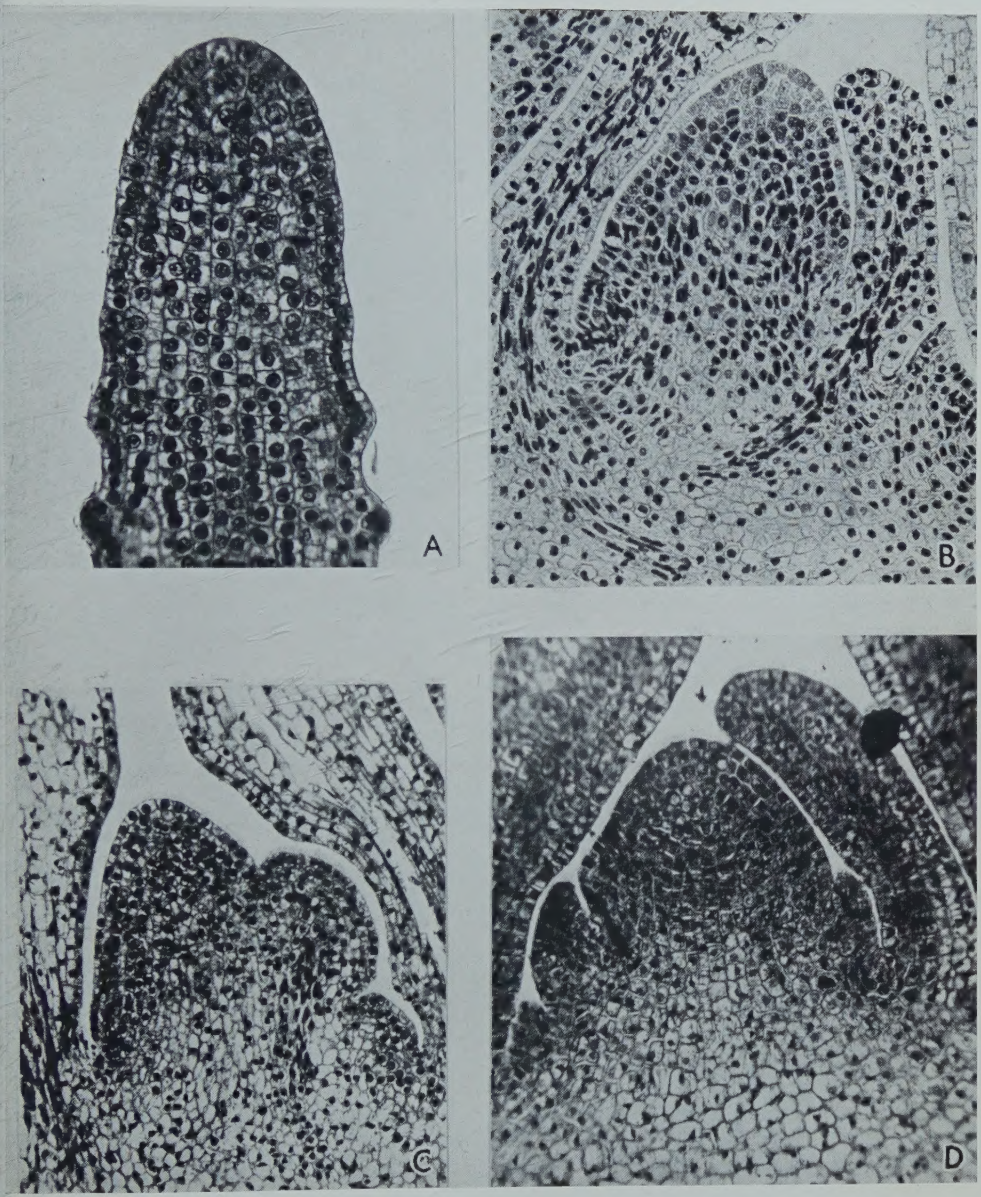
Illustrating Margaret Y. Stant's article on 'The Shoot Apex of Some Monocotyledons. I. Structure and Development'.

A. *Elodea densa.* L.S. growing-point showing files of rib meristem with larger initial cells at tip. Uniseriate tunica and anticlinal divisions associated with leaf initiation. (×260.)

B. *Convallaria majalis.* L.S. growing-point and first leaf primordia. Periclinal divisions in flank meristem immediately below tunica. Procambial strands visible. (×230.)

C. *Narcissus pseudo-narcissus.* L.S. shoot apex with three leaf primordia visible. Four tunica layers. First leaf primordium well developed on one side with procambial strand. (×120.)

D. *Luzula sylvatica.* L.S. shoot apex showing growing point and three youngest leaf primordia. Biseriate tunica. Corpus initials lightly stained and initiating the rib meristem by cambial-like divisions. (×350.)



M. STANT